CALMODULIN AND MALIGNANT HYPERPYREXIA

by

STEPHEN PETER COLLINS

A thesis submitted for the degree of Doctor of Philosophy
of the Australian National University, October 1984

STATEMENT

The investigations described in this thesis are my own original work.

Stephen Collins.

Stephen Collins

October, 1984

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr M.A. Denborough and Dr M.D. White for their very helpful advice, suggestions and discussions and for their assistance in the preparation of this thesis.

I also thank my departmental colleagues including Dr L. Marjanen, Dr G. Galloway, Mr K. Hopkinson, Mr A. Sim and Mr P. Foster for their helpful advice and discussions, and Mr K. Hopkinson for developing the computer programs for data retrieval. Thanks are also due to the staff of the library, the photography department and the animal house.

I gratefully acknowledge the support of an A.N.U. Ph.D Scholarship in the Department of Medicine and Clinical Science, John Curtin School of Medical Research.

Finally, I would like to thank my family for their support and encouragement.

ABSTRACT

The inherited anaesthetic complication Malignant Hyperpyrexia (MH) is thought to be due to an abnormality of skeletal muscle Ca^{2+} regulation. Because of this the Ca^{2+}-dependent regulatory protein calmodulin was investigated as a possible cause of abnormal Ca^{2+} regulation in MH susceptible (MHS) porcine muscle. Calmodulin is known to regulate intracellular Ca^{2+} concentrations and to modulate the effects of Ca^{2+} in many tissues.

The calmodulin antagonist drugs were shown to affect the contractile characteristics of both control and MHS porcine skeletal muscle in vitro. These drugs induced contracture in control and MHS muscle. Calmodulin antagonists also induced MH-like hypercontractility in control muscle and potentiated the hypercontractility of MHS muscle. These effects were consistent with the calmodulin antagonist drugs inducing an increase in myoplasmic Ca^{2+} concentration in both control and MHS muscle. Dantrolene sodium was able to partially reverse contractures induced by calmodulin antagonist drugs in both control and MHS porcine muscle.

The protein calmodulin was isolated from control and MHS porcine brain by an affinity chromatography procedure. Control and MHS calmodulins behaved identically during the isolation procedure and were indistinguishable by gel electrophoresis or by their UV absorbance spectra. The ability of control and MHS calmodulins to activate phosphodiesterase and to be inhibited by antagonist drugs were also identical. It was concluded that control and MHS calmodulins did not differ.

The sarcoplasmic reticulum (SR) of control and MHS porcine skeletal muscle was investigated as a possible site of action of the calmodulin antagonist drugs. These drugs inhibited the Ca^{2+}-dependent ATPase activity of isolated SR preparations in a dose-dependent manner. The extent of this inhibition was the same in both control and MHS SR. Calmodulin antagonist drugs inhibited the ATP-dependent Ca^{2+} uptake activity of isolated SR preparations from both control and MHS muscle to a similar extent. Also, the efflux of Ca^{2+} from isolated SR preparations was accelerated by calmodulin antagonist drugs. The extent of this acceleration was the same in both control and MHS SR. These effects of calmodulin antagonist drugs on SR Ca^{2+} transport functions were consistent with their in vitro pharmacological effects on control and MHS skeletal muscle. That is, these drugs affected the contractile characteristics of control and MHS muscle by raising the myoplasmic Ca^{2+} concentration.

Dantrolene sodium was unable to reverse the effects of the calmodulin antagonist drugs on brain calmodulin activity or skeletal muscle SR Ca^{2+} transport functions of either control or MHS tissue. However dantrolene sodium did partially reverse the contractures induced by calmodulin antagonist drugs in vitro. Since dantrolene sodium is thought to act on the excitation-contraction coupling mechanism, these observations suggested that the calmodulin antagonist drugs were acting, in part, on this mechanism to induce the changes in contractile characteristics observed in both control and MHS muscle in vitro.

Many of the calmodulin antagonist drugs used in the present investigation are clinically useful antipsychotics. The in vitro effects of these drugs, that is, the increasing of myoplasmic Ca^{2+}

concentrations, may be involved in two *in vivo* side effects of antipsychotic therapy. Firstly, patients undergoing antipsychotic therapy are at risk from heat stroke in high environmental temperatures. Secondly, psychiatric patients may experience a rare and dramatic side effect of antipsychotic therapy, the Neuroleptic Malignant Syndrome (NMS). The symptoms of NMS are similar to those of MH. Diagnostic studies in the present investigation and elsewhere have shown that NMS may be associated with MH.

The observations on the effects of calmodulin antagonist drugs on control and MHS porcine skeletal muscle strengthen the hypothesis that MH may be due to an abnormally high myoplasmic Ca^{2+} concentration. Calmodulin antagonist drugs raised myoplasmic Ca^{2+} concentrations by their effects on the SR Ca^{2+} transport functions and excitation-contraction coupling, thereby inducing MH-like hypercontractility in control muscle *in vitro*. An analysis of the effects of individual calmodulin antagonist drugs on control and MHS skeletal muscle implicated the excitation-contraction coupling mechanism as the site of the basic abnormality in porcine MH.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Ca^{2+}	calcium
CPK	creatine phosphokinase
CPM	counts per minute
CPZ	chlorpromazine
cyclic-AMP	cyclic-adenosine monophosphate
DEAE-cellulose	diethylaminoethyl-cellulose
DMSO	dimethylsulfoxide
E-C coupling	excitation-contraction coupling
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycol-bis-(β-amino-ethylether)-N,N'-tetraacetate
FPZ	fluphenazine
FSR	fragmented sarcoplasmic reticulum
HPD	haloperidol
I_{50}	concentration producing 50% inhibition
Kd	dissociation constant
ma	milliamperes
MH	malignant hyperpyrexia
MHS	malignant hyperpyrexia susceptible
MW	molecular weight
PEN	penfluridol
P_i	inorganic phosphate
PIM	pimozide
PMSF	phenylmethylsulfonylfluoride
PRO	promethazine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.D.	standard deviation
S.E.	standard error
SR	sarcoplasmic reticulum
TCA	trichloroacetic acid
TFP	trifluoperazine
Tris	tris (hydroxymethyl) aminomethane

CHAPTER 1 INTRODUCTION

1.1 The Malignant Hyperprexia Syndrome

Malignant Hyperpyrexia (MH) was first described in 1960 in a family in which 10 members had died during general anaesthesia (Denborough and Lovell, 1960). The propositus had exhibited a drastic reaction to anaesthesia given for the reduction of a compound fracture but had survived. Since that time MH has been recognized as an inherited complication of general anaesthesia. Susceptible individuals have a skeletal muscle abnormality which is associated with at least three clinically defined myopathies (King, Denborough and Zapf, 1972; Denborough, Dennett and Anderson, 1973; Moulds and Denborough, 1974d). Estimates of the incidence of MH put it at approximately 1/14,000 general anaesthetics (McPherson and Taylor, 1982).

1.2 Clinical Features of Malignant Hyperpyrexia

The onset of a MH episode is often sudden, either during or a short time after anaesthesia (Denborough, 1980). The early signs may include tachycardia, a falling blood pressure, arrhythmias, tachypnoea, sweating and cyanosis of the skin (Nissen and Yonkers, 1982). Muscle rigidity is present in 70-80% of cases. Later a rapid and sustained rise in body temperature is observed. Serum electrolyte estimations reveal a high potassium level and blood gas analysis shows high carbon dioxide output. Serum estimations show massive increases in muscle enzyme levels such as creatine phosphokinase (CPK), lactate dehydrogenase (LDH), glutamic oxalic transaminase (GOT) and hydroxybutyryl dehydrogenase (HBDH) (Britt, 1979). Late complications include disseminated intravascular

coagulation, myoglobinuria and renal failure. A mortality rate of 60-70% is observed.

1.3 Anaesthetic Triggers of Malignant Hyperpyrexia

Most potent inhalational anaesthetics will induce MH in susceptible individuals. These include halothane, methoxyflurane, diethyl ether, ethylchloride, trichloroethylene, cyclopropane and ethylene. The muscle relaxants succinylcholine and gallamine are also triggering agents for MH.

Safe anaesthesia is possible in MH susceptible (MHS) patients by the use of local, regional or spinal anaesthesia, or thiopentone, nitrous oxide, d-tubocurarine, belladonna alkaloids and althesin (Denborough, 1980).

1.4 Treatment of Malignant Hyperpyrexia

Fortunately, there is a drug available which has the ability to prevent or reverse a MH episode, the muscle relaxant dantrolene sodium (DANTRIUM, Norwich Pharmaceuticals) (Ellis and Bryant, 1972; Kolb, Horne and Martz, 1982). When an MH episode occurs during surgery, anaesthesia should be discontinued and the operation terminated. Dantrolene sodium should be given intravenously starting with an initial dose of 1-1.5 mg/kg, and repeated up to a cumulative dose of 10 mg/kg as long as the symptoms of MH persist (Aldrete, 1981).

Cooling, hyperventilation with oxygen and correction of acidosis and electrolyte abnormalities are also important early steps in the

treatment of MH. Cooling can be accomplished by immersion in ice and water baths. Metabolic acidosis can be treated with sodium bicarbonate. Diuretics such as mannitol help prevent hypernatremia from large quantities of sodium bicarbonate. Cardiac arrhythmias can be controlled by correction of fluid and electrolyte imbalances, however cardiac activity should be monitored continuously. Hyperkalemia may be reversed using 50% dextrose with soluble insulin. Coagulation studies should also be performed; heparin can be used to control coagulation if it develops. Finally, it is important to monitor patients closely even after resolution of symptoms since fatal recurrences of MH have been reported as occuring several hours later (Nissen and Yonkers, 1982).

1.5 The Genetics of Malignant Hyperpyrexia

Three clinical myopathies predisposing to MH have been defined. The Evans myopathy is the most common myopathy predisposing to MH, accounting for approximately 50% of MHS individuals (King, Denborough and Zapf, 1972; Britt, Endrenyi, Peters, Kwong and Kadijevic, 1976). It is usually sub-clinical although some muscle wasting may occur, and is inherited as a Mendelian dominant characteristic (Denborough, Ebeling, King and Zapf, 1970; McPherson and Taylor, 1982). Affected individuals may display elevated serum CPK levels (Isaacs and Barlow, 1970).

The King-Denborough syndrome is usually found in boys and is probably inherited as a recessive characteristic (King and Denborough, 1973). The boys are usually small for their age and have undescended testes,

thoracic kyphosis, lumbar lordosis, pectus carinatum, low-set ears and ptosis.

Central-core disease is an uncommon myopathy and is often associated with MH (Denborough, Dennett and Anderson, 1973). This myopathy is characterized by striking 'core' lesions in muscle fibres on histological examination.

Although MH episodes have been reported in patients suffering from congenital muscular dystrophy (Fletcher, Blennow, Olsson, Ranklev and Tornebrant, 1982) and Duchenne's muscular dystrophy (Kelfer, Singer and Reynolds, 1983), pharmacological testing of muscle from patients with these and other muscle diseases does not show the hypercontractility which is characteristic of MH susceptibility (Moulds and Denborough, 1974d).

1.6 The Diagnosis of Malignant Hyperpyrexia

The most widely accepted and reliable method of diagnosing susceptibility to MH is the *in vitro* muscle contracture test (Kalow, Britt, Terreau and Haist, 1970; Ellis, Keaney, Harriman, Sumner, Kyei-Mensah, Tyrrell, Hargreaves, Parikh and Mulrooney, 1972; Moulds and Denborough, 1974b). This procedure necessitates the excision of a muscle biopsy specimen, usually from the vastus lateralis. Fibre bundles are cut from the specimen and suspended in a physiological solution, supplied with glucose and oxygen, and maintained at body temperature. The muscle strips are then exposed to 3% halothane, 2mM caffeine, 1mM succinylcholine (ANECTINE, Wellcome Australia Ltd.) and

80mM potassium chloride. Muscle strips from susceptible individuals show increased contracture responses at these concentrations.

Other in vitro methods for identifying susceptibility to MH using muscle biopsy specimens have been proposed. The ATP depletion test was based on the observation of a decrease in the ATP concentration in MH muscle exposed to halothane (Harrison, Saunders, Biebuyck, Hickman, Dent, Weaver and Torblanche, 1969). It was found, however, that this test did not identify all of the MHS population (Britt, Endrenyi, Kalow and Peters, 1976). An increase in myophosphorylase A in MH muscle has been proposed as a diagnostically useful test by Willner, Wood, Cerri and Britt (1980), but when this parameter was studied by another group (Traynor, Van Dyke and Gronert, 1983) an unacceptably high number of false positive responses were observed.

In the diagnosis of MH, muscle biopsy specimens are also taken for histopathological and electron microscopic examination. Not all MHS patients show muscle pathology and the changes are not specific. In a reported series of 80 MH patients 35 showed myopathic changes at biopsy (Harriman, 1982). The most frequent myopathic change was internal nuclei, and others included flattened fibres, small angular basophilic fibres, fibre splitting, moderate atrophy, moth-eaten and core-targetoid fibres.

The disadvantage of the above diagnostic methods is their invasive nature. A number of studies have been reported in which blood cells or serum enzymes have been investigated for their diagnostic value in MH. Platelet aggregation was shown to be normal in MHS individuals

(Rosenberg, Fisher, Reed and Addonizio, 1981). Studies of halothane-induced ATP depletion in platelets have produced conflicting results. One study reported a significant decrease of ATP in MHS platelets when compared with controls (Solomons, McDermott and Mahowald, 1980) but another study observed no difference (Giger and Kaplan, 1983). The most commonly studied serum enzyme in MH was CPK which was elevated in approximately 70% of MHS individuals (Blank and Gruener, 1983). Evaluation of CPK levels as a screening method for MH in an ambulatory surgical patient population showed a poor correlation between high serum CPK and susceptibility to MH (Amaranath, Lavin, Trusso and Boutros, 1983). Typing of the human leukocyte antigen in a single MHS family showed no diagnostic value (Lutsky, Witkowski and Henschel, 1982).

Anthropometric studies (that is, height, weight and skinfold thickness) have been shown to be of no diagnostic value in MH (Campbell, Ellis, Halsall and Hogge, 1982).

1.7 Syndromes Related to Malignant Hyperpyrexia

Malignant hyperpyrexia usually presents clinically in susceptible individuals when anaesthesia is induced with drugs which trigger MH. It may also present, however, as heat-stroke after severe physical stress (Denborough, 1982) or rhabdomyolysis after serious infections (Denborough, Collins and Hopkinson, 1984). In the case of heat-stroke, dantrolene sodium has been shown to be an effective treatment (Denborough, 1982; Lydiatt and Hill, 1981). An association between MH and the sudden infant death syndrome (SIDS) has also been described (Denborough, Galloway and Hopkinson, 1982). In this study

5 out of 15 parents whose children had died from SIDS were identified as MHS.

1.8 Malignant Hyperpyrexia in Animals

Malignant hyperpyrexia has been identified in pigs (Hall, Woolf, Bradley and Jolly, 1966), cats (De Jong, Heavner and Amory, 1974), dogs (Short and Paddleford, 1973) and horses (Williams, 1976). Porcine MH has become the animal model of human MH with *in vitro* pharmacological studies of skeletal muscle showing identical behaviour in both species (Okumura, Crocker and Denborough, 1979).

The MH susceptibility found in pigs is associated with two other conditions resulting from interbreeding. These are the porcine stress syndrome (PSS) and the pale, soft, exudative pork syndrome (PSEP). Porcine stress syndrome is induced by stresses such as fighting or transportation and its symptoms include shortness of breath, increasing body temperature, patchy cyanosis of the skin and collapse and death within a few minutes (Topel, Bicknell, Preston, Christian and Matsushima, 1968). Pale, soft, exudative pork syndrome refers to poor meat quality post-mortem and is a feature of fast glycolysing muscle which produces large quantities of lactate before the muscle can be cooled (Briskey, 1964). It appears that MH, PSS and PSEP are different manifestations of the same syndrome in swine (Nelson, 1973).

1.9 The Aetiology of Malignant Hyperpyrexia

The major precipitating event when a MHS individual or MHS pig is administered halothane appears to be a sudden rise in the myoplasmic

Ca^{2+} concentration (Britt, 1983; Denborough, 1980). The basic abnormality in MH muscle accounting for this rise has yet to be elucidated although a number of possibilities have been suggested. These included an abnormality of excitation-contraction coupling (E-C coupling) (Denborough, 1980; Okumura, Crocker and Denborough, 1980), an abnormality of Ca^{2+} uptake by the sarcoplasmic reticulum (SR) (Gronert, Heffron and Taylor, 1979; Blank, Gruener, Suffecool and Thompson, 1981), an abnormality of Ca^{2+} release by the SR (Nelson, 1983; Ohnishi, Taylor and Gronert, 1983), defective Ca^{2+} storage by the mitochondria (Cheah and Cheah, 1978, 1979), or an increased adenylate cyclase activity with a resulting increase in cyclic-AMP levels (Willner, Cerri and Wood, 1981).

Whatever the basic cause, a rise in myoplasmic Ca^{2+} will have multiple heat-producing effects. Phosphorylase will become activated leading to the breakdown of glycogen to lactic acid, carbon dioxide and heat. Actomyosin ATPase will become activated and hydrolyze ATP to ADP, phosphate, heat, and energy which is utilized in muscle contraction. Ca^{2+} will also bind to troponin C initiating contraction. The elevated levels of Ca^{2+} reached in MHS muscle during a MH episode cause further problems in the mitochondria. Oxidative phosphorylation is uncoupled from electron transport, thereby decreasing ATP production and further exacerbating oxygen consumption and lactate, carbon dioxide and heat output. Muscle ATP levels fall rapidly once the stores of high energy creatine phosphate have been exhausted, and enzymes utilizing ATP as an energy source no longer function efficiently. These enzymes include the sarcolemmal ATPases which ensure a favourable intracellular environment. Ions

and molecules diffuse into and out of the muscle cell. Calcium ions flow into the cell further exacerbating the situation. Once the sarcolemma has been breached, enzymes and myoglobin leak outward, and myoglobin can obstruct the renal tubules and cause renal failure. The changes produced in serum pH and electrolyte balance can act on body tissues and cause haemolysis, consumption coagulopathy, and cardiac arrhythmias (Britt, 1983; Denborough, 1980).

1.10 The Site of the Basic Abnormality in Malignant Hyperpyrexia

1.10.1 Skeletal Muscle and Malignant Hyperpyrexia

It is accepted that skeletal muscle is the site of the basic abnormality in MHS individuals. This belief was based, in the first instance, on a number of clinical observations of MH episodes. During a MH crisis the skeletal musculature of many patients displays a generalized rigidity. The serum levels of muscle specific enzymes such as CPK show dramatic increases, indicating muscle damage (Britt, 1979). Also the observation that in the face of generalized rigidity during a MH episode a limb to which a tourniquet had been applied early in anaethesia remained flaccid, indicated that the anaesthetic was affecting skeletal muscle (Drury and Gilbertson, 1970). It was also found that some MHS individuals showed clinically discernable myopathies (Denborough, Ebeling, King and Zapf, 1970; King, Denborough and Zapf, 1972; Denborough, Dennett and Anderson, 1973; Moulds and Denborough, 1974d) and that approximately 70% of susceptible individuals have an elevated resting serum CPK (Isaacs and Barlow, 1970). Following these observations, *in vitro* pharmacological studies of skeletal muscle from MHS individuals have shown an abnormal contractility of this tissue (Kalow, Britt, Terreau

and Haist, 1970; Ellis, Keaney, Harriman, Sumner, Kyei-Mensah, Tyrrell, Hargreaves, Parikh and Mulrooney, 1972; Moulds and Denborough, 1974a). Before going on to describe the pharmacological and biochemical findings in MH muscle the structure and biochemistry of skeletal muscle will be briefly reviewed.

1.10.2 Structural Organisation of Skeletal Muscle

1.10.2.1 Macroscopic Organization

Skeletal muscle accounts for 40-50% of total mammalian body weight, and its specialized function is to convert chemical energy into contractile force. The muscle consists of a large number of muscle fibres which are elongated, multinucleate cells running from one end of a muscle to the other. These fibres can be 10 cm or more in length and their diameters range from 10-100 μm. Individual fibres are separated from their neighbours by a fine network of collagen fibrils called the endomysium. Groups of muscle fibres, or fascicles, are divided from one another by another collagen layer called the perimysium, and finally the whole muscle is enclosed in a strong collagen coat, the epimysium.

1.10.2.2 Microscopic Organization

The plasma membrane of a muscle fibre is referred to as the sarcolemma. The contractile elements, the myofibrils, take up 85-90% of the fibre volume. They are surrounded and bathed by the sarcoplasm, the intracellular fluid of muscle. The sarcoplasm contains glycogen, glycolytic enzymes, ATP, phosphocreatine and inorganic electrolytes, as well as a number of amino acids and peptides. Mitochondria, which are very profuse in more active

muscles, are regularly arranged along the myofibrils. The muscle fibre also contains a highly differentiated endoplasmic reticulum known as the sarcoplasmic reticulum (SR) which functions in the control of contraction as a store of Ca^{2+} (Figure 1.1). The t-tubules, invaginations of the sarcolemma, form junctions with the terminal cisternae of the SR (triad junctions) (Figure 1.1).

The myofibrils are irregular polygons in cross-section with a mean diameter of 1 μm and of indefinite length. They are composed of serially repeating segments, the sarcomeres, the lateral alignment of which gives rise to the characteristic cross striations of the whole fibre (Figure 1.2). The light bands are called isotropic or I bands, and the dark bands are called anisotropic or A bands. In resting muscle, the A bands are approximately 1.6 μm long and the I bands approximately 1 μm long. The I bands are bisected by a dense transverse line about 800 Å wide known as the Z line. The central portion of the A band, approximately 0.5 μm wide, called the H zone and less dense than the rest of the A band, is bisected by another dense, transverse line, the M line. The serially repeated unit, the sarcomere, extends from Z line to Z line (Figure 1.2) (Landon, 1982).

Electronmicroscopic examination of the myofibril in cross-section has shown that it is made up of many parallel myofilaments (Huxley and Hansen, 1954). The H zone has a hexagonal array of thick filaments (15-18 nm in diameter). The I band, on the other hand, displays a less regular array of thin filaments (7nm in diameter). In the denser parts of the A band both filament types are present, each thick filament surrounded by six thin filaments (Figure 1.2). The

Figure 1.1 Schematic Diagram of Muscle Cell Structure From Bloom and Fawcett (1975).

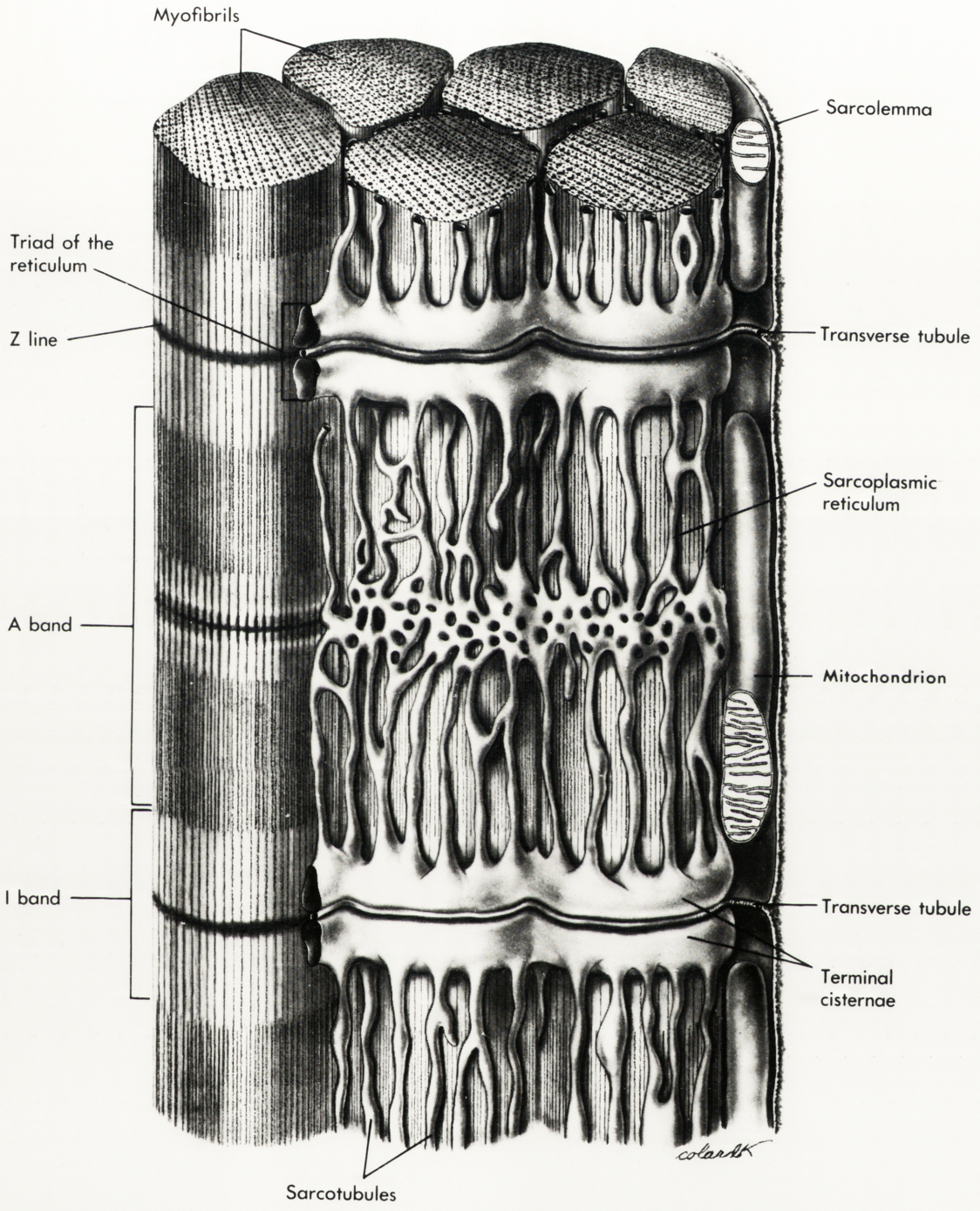
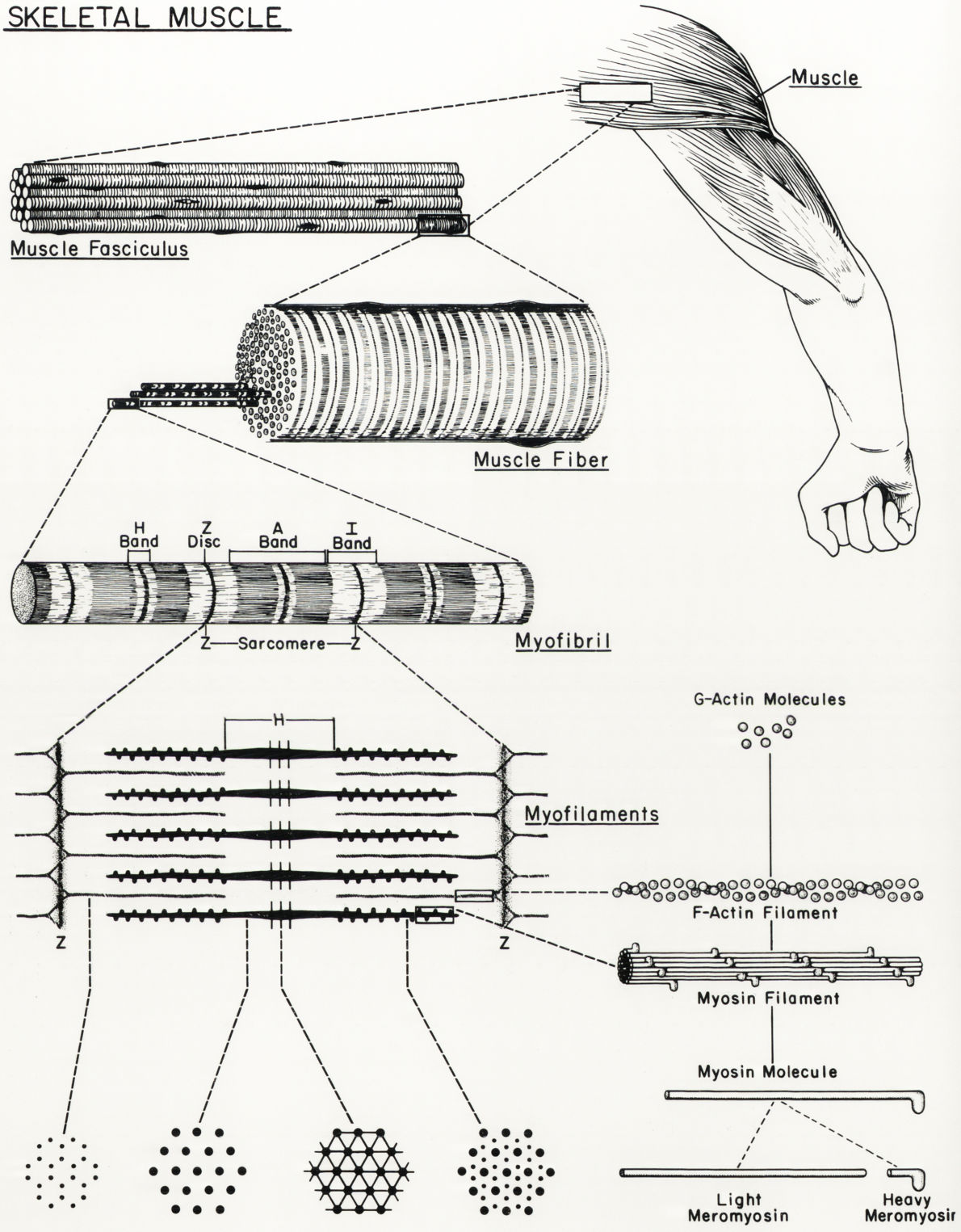

Myofibrils
Sarcolemma
Triad of the reticulum
Z line
Transverse tubule
Sarcoplasmic reticulum
A band
Mitochondrion
I band
Transverse tubule
Terminal cisternae
Sarcotubules

Figure 1.2 Diagram of the Structure of the Myofibrils. From Bloom and Fawcett, (1975).


SKELETAL MUSCLE
Muscle
Muscle Fasciculus
Muscle Fiber
H Band
Z Disc
A Band
I Band
Z—Sarcomere—Z
Myofibril
H
Myofilaments
Z
Z
G-Actin Molecules
F-Actin Filament
Myosin Filament
Myosin Molecule
Light Meromyosin
Heavy Meromyosin

thick filaments extend throughout the A band, and the thin filaments extend from the Z line to the H zone boundary. The overlap of the two filament types gives rise to the denser parts of the A band. In these denser regions regularly disposed cross-bridges between the thick filaments and adjacent thin filaments have been observed. These cross-bridges project from the thick filaments (Sjostrom and Squire, 1977). During contraction the I band and H zone are shortened while the A band length remains unchanged. Observations such as these led to the formulation of the sliding-filament theory of muscle contraction (Huxley and Hansen, 1954; Huxley and Niedergerke, 1954). In this theory, changes in the length of a muscle are due to the sliding of the thick and thin filaments past each other due to their interaction through the cross-bridges.

1.10.2.3 Molecular Organization

The thick filaments are composed almost entirely of the protein myosin. The myosin molecule is rod shaped (approximately 150 nm long) with a molecular weight (MW) of 470,000. It is made up of two strands forming an alpha-helix and bears two pear-shaped heads at one end (Elliot and Offer, 1978). The shafts of the myosin molecules stack together to form the shaft of the thick filament in such a manner that the paired heads lie on its surface, and the myosin molecules in each half of each thick filament are arrayed with opposite polarities (Figure 1.3a) (Huxley, 1963). The myosin heads correspond to the crossbridges seen between the thick and the thin filaments. The thin filaments are composed mainly of actin. The actin monomer (G actin) is a globular molecule (diameter 5.46 nm) with a MW of 45,000. These monomers form a filamentous polymer (F

actin), a right handed, two stranded helix (diameter 6-7 nm) twisted so that there are 13-15 G actin molecules for every full rotation of the helix (Huxley and Brown, 1967). A second thin filament protein, tropomyosin, lies in the grooves on either side of the two chains of actin molecules (Figure 1.3b). Tropomyosin is a rod shaped, left-handed alpha-helix 38.5 nm long with a MW of 63,000. These molecules lie head to tail along the length of the thin filament (Cohen, Caspar, Parry and Lucas, 1971; Ebashi, 1980). The third thin filament protein is troponin. This structure is globular with a MW of about 80,000 (Ebashi, Kodama and Ebashi, 1968). One troponin unit is attached to each tropomyosin molecule to give a periodicity of 38.5 nm along the thin filament (Ohtsuki, 1975). Troponin itself is made up of three subunits, I, C and T. Troponin T binds the structure to tropomyosin, troponin I inhibits the interaction of the thick filament cross-bridges with the thin filament, and troponin C confers Ca^{2+} sensitivity on this inhibition (Potter and Gergely, 1974). The binding of Ca^{2+} to troponin C allows interaction between the thick and thin filaments, and thus allows contraction to proceed (assuming energy is available).

1.10.3 The Control of Contraction in Skeletal Muscle

In resting muscle the Ca^{2+} concentration in the sarcoplasm is in the range 10^{-7}-10^{-8} M (Ashley, 1978), intracellular Ca^{2+} being sequestered by the SR. This concentration gradient is upheld by a Ca^{2+}-dependent transport ATPase present in the SR membrane. The Ca^{2+} is stored within the SR bound to a high capacity, low affinity Ca^{2+} binding protein, calsequestrin (Otswald and MacLennan, 1974). When

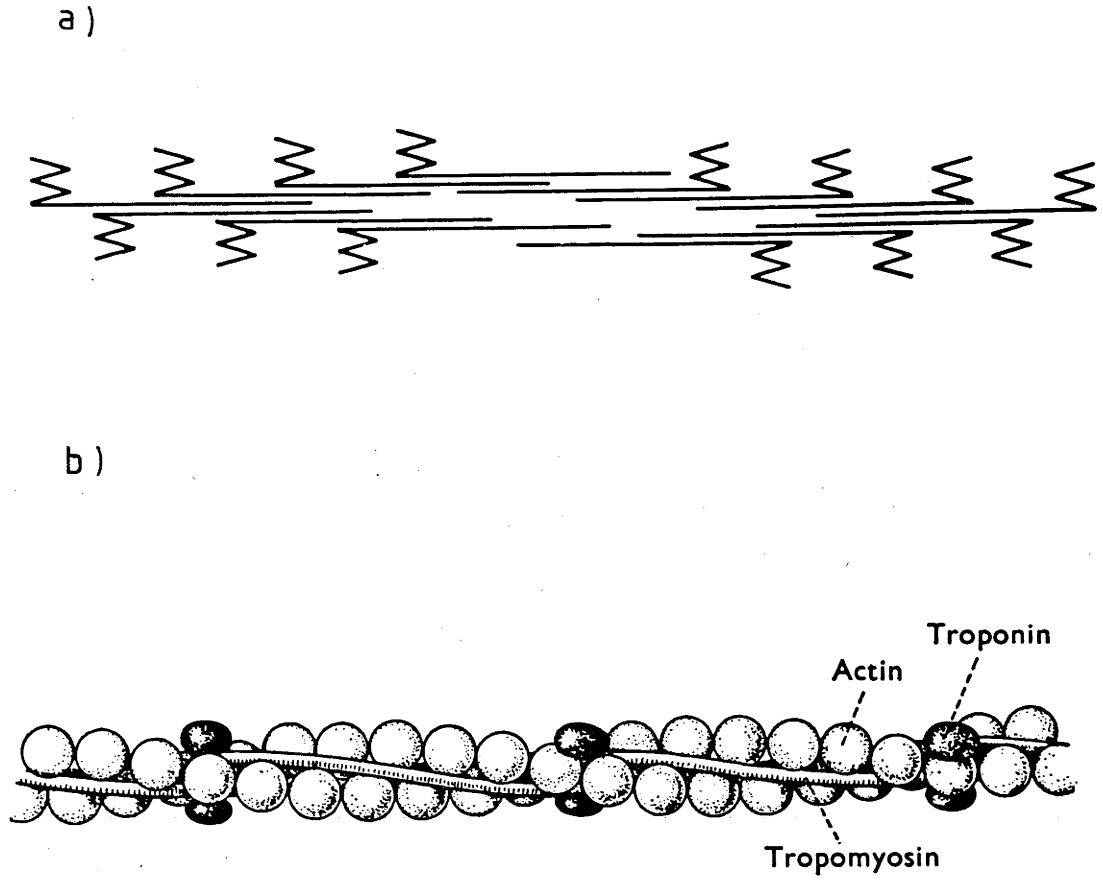


Figure 1.3 Schematic Diagram of Thick and Thin Filament Structure.

a) Thick filament composed of myosin molecules.

b) Thin filament composed of actin, tropomyosin and troponin.

From Bourne (1972).

the innervating motor-neuron stimulates the muscle cell to contract (that is, releases acetylcholine at the motor endplate) the sarcolemma becomes depolarized. This transient depolarization (from a resting level of -90 mV to -50 mV), resulting from a voltage and time-dependent increase in sodium conductance, spreads over the sarcolemma and t-tubules into the interior of the fibre (Nastuk and Hodgkin, 1950). Here the t-tubules form their triadic junctions with the SR (Figure 1.1) and through an unknown coupling mechanism stimulate the SR to release its stored Ca^{2+} (Costantin, 1975). When the nervous stimulation ceases, release ceases, the Ca^{2+}-dependent transport ATPase can again reduce the sarcoplasmic Ca^{2+} concentration and the fibre relaxes (Martonosi, 1982).

The nature of the coupling between the t-tubule and the SR at the triad junction (excitation-contraction or E-C coupling) has received much investigation. It is known that the junctional gap (100-200 Å wide) is bridged by regularly disposed densities termed SR feet (Franzini-Armstrong, 1980). Schneider and Chandler (1973) proposed that charge movement within the t-tubule membrane directly effects the SR membrane permeability to Ca^{2+}, the feet acting as the link between the two. Mathias, Levis and Eixenberg (1980), on the other hand, envisaged a permeability channel spanning the entire junctional gap. The t-tubule depolarization led to its transitory opening and subsequently to the initiation of a voltage-dependent increase in permeability to Ca^{2+} of the SR membrane. Other theories of E-C coupling in skeletal muscle provide no explicit role for the SR feet. The most important among these is the 'trigger' Ca^{2+} theory (Endo, Tanaka and Ogawa, 1970; Frank, 1982). This theory holds that a

release of a small amount of Ca^{2+} from the t-tubule, either bound to the membrane surface or within the tubule, leads to the release of larger amounts of Ca^{2+} from the SR itself, thus initiating contraction.

1.10.4 The Source of Energy for Contraction in Skeletal Muscle

The energy required by skeletal muscle to generate contractile force is provided by ATP. The sliding of the thick and thin filaments past one another is accomplished by the cyclical attachment and release of the cross-bridges and is driven by an ATPase enzyme within the myosin molecule (Huxley, 1962). The amount of ATP stored in muscle can sustain contraction for only a fraction of a second. High energy phosphoryl groups are stored in the form of phosphocreatine. This compound replenishes the ATP supply through the action of CPK.

$$\text{Phosphocreatine} + \text{ADP} \rightleftharpoons \text{ATP} + \text{creatine}$$

As phosphocreatine stores are depleted the level of ATP falls and the levels of ADP and P_i rise. The level of AMP also rises through the action of adenylate kinase.

$$2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$$

The resulting fall in the muscle cell energy charge stimulates glycolysis, the citric acid cycle and oxidative phosphorylation. The relative contribution of these pathways to ATP generation depends on the type of muscle examined.

1.10.5 Dantrolene Sodium and Skeletal Muscle

Dantrolene sodium (DANTRIUM, Norwich Pharmaceuticals) is a lipid soluble hydantoin derivative (Snyder, Davis, Bickerton and Halliday,

1967). It is a skeletal muscle relaxant which acts directly on muscle (Ellis, Castellion, Honkomp, Wessels, Carpenter and Halliday, 1973) and is thought to inhibit E-C coupling (Ellis and Bryant, 1972). Dantrolene sodium has been shown to lower resting myoplasmic Ca^{2+} concentrations and to inhibit the release of Ca^{2+} from the SR in intact cells, (Desmedt and Hainaut, 1977, 1979), but it did not stimulate the Ca^{2+}-dependent ATPase of isolated SR or affect Ca^{2+} exchange or efflux rates of isolated SR (White, Collins and Denborough, 1983).

1.11 Calmodulin

1.11.1 Introduction

Over 40 years ago Heilbrunn (1943) considered that Ca^{2+} played a central role in almost every physiological process. Although today it is known that Ca^{2+} does not play as universal a role as Heilbrunn suggested, Ca^{2+} exerts a profound influence on many biological processes such as cell motility, muscle contraction, axonal flow, cytoplasmic streaming, chromosome movement, neurotransmitter release, endocytosis and exocytosis. Since 1970 it has become clear that many of the cellular effects of Ca^{2+} are mediated through a primary receptor protein, the Ca^{2+}-dependent modulator protein, calmodulin.

1.11.2 Biological and Physiochemical Properties of Calmodulin

Calmodulin is a small, monomeric protein composed of 148 amino acids (MW approximately 17,000) (Watterson, Sharief and Vanaman, 1980). It is a highly conserved protein occurring in all the eukaryotic cells that have been examined (Cheung, 1980). Calmodulins isolated from various vertebrate sources and tissues appear to be identical

proteins (Watterson, Mendel and Vanaman, 1980; Kakiuchi, Sobue, Yamazaki, Kambayashi, Sakon and Kosaki, 1981). Plant and invertebrate calmodulins show only minor variations in amino acid composition (Grand, Nairn and Perry, 1980; Yasawa, Sakuma and Yagi, 1980). Calmodulin has a high content of acidic amino acids (isoelectric point approximately 4.0) and a trimethylated lysine residue at position one hundred and fifteen (Watterson, Sharief and Vanaman, 1980). Another property of calmodulin, which is frequently exploited in isolation procedures, is its remarkable heat stability (Scharff, 1981).

Calmodulin appears to be related to other Ca^{2+}-binding proteins such as troponin C and parvalbumin. The conservative replacement sequence homology between calmodulin and troponin C is approximately 70% and is particularly strong between the Ca^{2+} binding domains of these two proteins (Dedman, Jackson, Schreiber and Means, 1978).

Calmodulin was first identified as a Ca^{2+}-dependent activator protein of a brain 3'-5' cyclic nucleotide phosphodiesterase (Cheung, 1980). Since that time it has been found to function in many cellular processes as a mediator of Ca^{2+} effects (Table 1.1). Calmodulin by itself is not biologically active. The active form is the Ca^{2+}-calmodulin complex with calmodulin having four Ca^{2+} binding sites (Wolff, Poirier, Brostrom and Brostrom, 1977). These Ca^{2+} binding sites share a common protein backbone configuration with the Ca^{2+} binding sites of troponin C and parvalbumin which was termed the "E-F hand" by Kretsinger and Barry (1975; Kretsinger, 1980). This Ca^{2+} binding, which has been reported as cooperative (Crouch and

Table 1.1 Cellular Functions in which Calmodulin has been Implicated

Intracellular Ca^{2+} regulation	
Ca^{2+}-ATPase - erythrocyte plasma membrane	Roufogalis, 1979
- cardiac SR	Katz and Remtulla, 1978
Ca^{2+} buffering	Vincenzi, 1979
Skeletal muscle SR protein phosphorylation	Chiesi and Carafoli, 1983
Contractile Systems	
Myosin light chain kinase - skeletal muscle	Crouch, Holroyde, Collins, Solaro and Potter, 1981
- smooth muscle	Cheung, 1980
Microfilaments	Dedman, Brinkley and Means, 1979
Microtubules	Dedman, Brinkley and Means, 1979
Cilia	Jamieson, Vanaman and Blum, 1979
Cyclic nucleotide metabolism	
Phosphodiesterase	Cheung, 1980
Adenylate cyclase	Wang and Waisman, 1979
Guanylate cyclase	Cheung, 1980
Glycogen metabolism	
Glycogen synthase kinase	Klee, Crouch and Richman, 1980
Phosphorylase kinase	Klee, Crouch and Richman, 1980
Neural tissue	
Neurotransmitter release	Cheung, 1980
Postsynaptic	Cheung, 1980
Axoplasmic transport	Iqbal and Ochs, 1980
Calmodulin binding proteins	Wang and Waisman, 1979

Lipid metabolism	
Phospholipase A_2	Wong and Cheung, 1979
Phospholipid transmethylation	Gil, Alemany, Cao, Castano and Mato, 1980
Mitosis	
Synthesis of DNA	Boynton, Whitfield and MacManus, 1980
Microtubules	Dedman, Brinkley and Means, 1979
Ca^{2+}-ATPase	Dedman, Brinkley and Means, 1979
Other	
NAD kinase	Klee, Crouch and Richman, 1980
Platelet function	White and Raynor, 1980
Secretion of intestinal ions	Ilundain and Naftalin, 1979
Tryptophan 5'-monooxygenase	Yamauchi and Fujisawa, 1979a
Protein kinases - membrane	Schulman and Greengard, 1978
- cytosol	Yamauchi and Fujisawa, 1979b

Klee, 1980), induced a conformational change exposing a hydrophobic surface on the calmodulin molecule (La Porte, Wierman and Storm, 1980). This Ca^{2+}-induced hydrophobic site may be the means by which calmodulin binds to its effector proteins and thereby exerts its action. In some cases calmodulin exerts its Ca^{2+}-dependent effects as a non-dissociable subunit of an enzyme, for example, phosphorylase kinase of skeletal muscle (Cohen, Burchell, Foulkes, Cohen, Vanaman and Nairn, 1978).

Some of the cellular functions with which calmodulin has been identified are shown in Table 1.1. Not all cells will express all of these functions simultaneously. Coordinate modulation of calmodulin-dependent functions might be achieved in a number of ways including variations in the affinity of the effector proteins of calmodulin for the Ca^{2+}-calmodulin complex. The occupancy state of the four Ca^{2+} binding sites may also be important (Crouch and Klee, 1980). The concentration of the Ca^{2+}-calmodulin complex itself is affected by fluctuations in the levels of cellular Ca^{2+}, and perhaps by variations in the rates of synthesis and/or degradation of calmodulin. Cyclic-AMP-dependent phosphorylation of membranes induced the release of calmodulin from the membrane (Hanbauer, Pradham and Yang, 1980) and also increased the binding of calmodulin to membrane-bound phosphodiesterase (Clayberger, Goodman and Rasmussen, 1981). The modulation of cell function by Ca^{2+} and its interactions with cyclic-AMP mediated systems have been extensively reviewed by Rasmussen and Waisman (1983).

1.11.3 Calmodulin in Skeletal Muscle

Calmodulin is present in skeletal muscle at a concentration of approximately 3 µg/g tissue (Yagi, Yazawa, Kakiuchi, Oshima and Uenishi, 1978). Immunocytochemical techniques have shown that it is localized mainly at the level of the I bands and in the terminal cisternae (Harper, Cheung, Wallace, Levine and Steiner, 1980). Unlike cardiac SR, however, regulation of the Ca^{2+}-ATPase of skeletal muscle SR did not appear to involve calmodulin (Chiesi and Carafoli, 1982). The specific function of calmodulin in skeletal muscle appears to be the regulation of Ca^{2+}-dependent protein phosphorylation. Calmodulin has been shown to activate three protein kinases in this tissue, phosphorylase kinase, myosin light chain kinase (MLCK), and an undefined protein phosphorylation system that has been recently described (Campbell and MacLennan, 1982).

Phosphorylase kinase is involved in the metabolism of the muscle glycogen stores. It activates glycogen phosphorylase, the enzyme which liberates glucose-1-phosphate from glycogen. Phosphorylase kinase phosphorylates the inactive phosphorylase b to the active phosphorylase a. This kinase activity is modulated by Ca^{2+}-levels through calmodulin which is bound as a non-dissociable subunit of the enzyme (Cohen, Burchell, Foulkes, Cohen, Vanaman and Nairn, 1978). In rabbit skeletal muscle the regulation of the red muscle isozyme of phosphorylase kinase by Ca^{2+} was mediated by the tightly bound calmodulin subunit and was insensitive to exogenous calmodulin. The white muscle phosphorylase kinase isozyme was also regulated by Ca^{2+} through its calmodulin subunit, but exogenous calmodulin provided additional Ca^{2+}-dependent activation of the enzyme (Wang, Tam, Lewis

and Sharma, 1981). This additional activation of phosphorylase kinase in white muscle (fast twitch) may help in the quick response to increases in cellular Ca^{2+} needed in fast muscle contraction.

Myosin light chain kinase is responsible for the phosphorylation of a low MW component of myosin (Mayr and Heilmeyer, 1983). This component is one of three myosin "light chains" which can be separated from native myosin by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) (Perrie, Smillie and Perry, 1973), and is referred to as $M1_2$, LC_2, g_2 or P light chain. In smooth muscle the phosphorylation of this light chain is essential for contraction (Sparrow, Mrwa, Hofmann and Ruegg, 1981). In skeletal muscle, however, this is not the case and a role for MLCK phosphorylation of the light chain has not been found. Cycles of phosphorylation and dephosphorylation of this light chain have been observed in response to contraction and relaxation in whole skeletal muscle (Manning and Stull, 1979). Crow and Kushnerick (1982) have proposed that light chain phosphorylation may regulate the rate of cross-bridge turnover, but Butler, Siegman, Mooers and Barsotti (1983) disputed this. Whatever the function of MLCK, it is regulated by calmodulin. In the absence of calmodulin MLCK is inactive, while in the presence of 1 mole of calmodulin/mole of MLCK, 80-90% of maximum activity is obtained (Perry, Grand,Nairn,Vanaman and Wall, 1979).

The third and most recently described calmodulin-dependent protein kinase system in skeletal muscle is located in the SR membrane. Campbell and MacLennan (1982) reported the calmodulin-mediated

phosphorylation of two SR proteins of MW 20,000 and 60,000. They concluded that this calmodulin-dependent phosphorylation may have some role in Ca^{2+} release from the SR. Chiesi and Carafoli (1982) found three SR proteins in which phosphorylation was promoted by calmodulin. The MW's of these proteins were 57,000, 35,000 and 20,000. These authors also found that calmodulin-mediated phosphorylation of these proteins had no effect on Ca^{2+} uptake by SR vesicles. They too concluded that this phosphorylation system may regulate Ca^{2+} release from the SR. Further investigation (Chiesi and Carafoli, 1983) showed that none of the three proteins interacted directly with calmodulin, and that the calmodulin-dependent protein kinases involved were membrane bound.

1.11.4 Pharmacological Antagonism of Calmodulin Function

It is possible to antagonize the actions of calmodulin by the use of certain classes of clinically used antipsychotic drugs (Levin and Weiss, 1979). More recently, other types of drugs (such as R24571, a derivative of an antimycotic) have been shown to be effective calmodulin antagonists (Table 1.2) (Gietzen, Wuthrich and Bader, 1981). These agents have been used extensively as probes to elucidate calmodulin functions and have proved to be a major development in the study of this Ca^{2+}-regulatory protein. However, as stated by Roufogalis, Minocherhomjee and Al-Jobore (1983), "currently available calmodulin antagonists are neither selective nor specific, despite their usefulness when used appropriately".

Trifluoperazine (TFP; STELAZINE, Smith Kline and French) (Figure 1.4) and related phenothiazines were shown to inhibit calmodulin-dependent

Table 1.2 Various Compounds which Antagonize Calmodulin Activity

COMPOUND	CLASS	I_{50} PDE*	I_{50} RBC-ATPase+	REF.
Trifluoperazine	Phenothiazine antipsychotic	10 μM		Levin and Weiss, 1979
Fluphenazine	"		10 μM	Roufogalis, 1982
Thioridazine	"	18 μM		Roufogalis, 1982
Chlorpromazine	"	42 μM		Levin and Weiss, 1979
Promethazine	"	340 μM		Weiss, Prozialeck and Wallace, 1982
Penfluridol	Diphenylbutylpiperidine antipsychotic	2.5 μM		Levin and Weiss, 1979
Haloperidol	Butyrophenone antipsychotic	60 μM		Levin and Weiss, 1979
Pimozide	Diphenylbutylamine antipsychotic	7 μM		Levin and Weiss, 1979
R24571	Antimycotic derivative		0.35 μM	Gietzen, Wuthrich and Bader, 1981
Diazepam	Minor tranquilizer	140 μM		Hidaka, Yamaki, Totsuka and Asano, 1979
Pentobarbital	Barbiturate anaesthetic	10 mM		Levin and Weiss, 1979
Dopamine	Central neurotransmitter	10 mM		Levin and Weiss, 1979
W-7	Smooth muscle relaxant	67 μM		Hidaka, Yamaki, Naka, Tanaka, Hayashi and Kobayashi, 1980
Sulpiride	Benzamide antipsychotic	100 μM		Norman, Drummond and Moser, 1979
B-endorphin	Peptide opiate	3 μM		Weiss, Prozialeck, Cimino, Barnette and Wallace, 1980
Triton X-100	Nonionic detergent	14 μM		Sharma and Wang, 1981

* calmodulin-activated phosphodiesterase activity
\+ calmodulin activated red blood cell ATPase activity

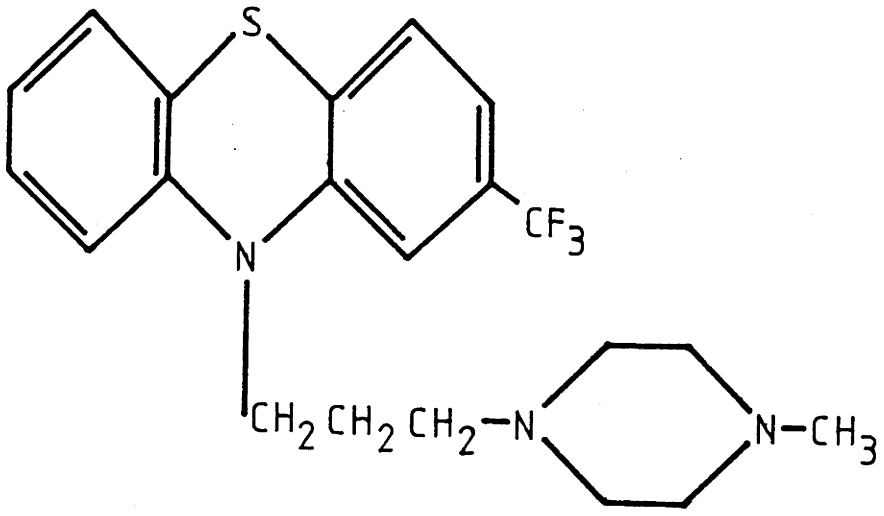


Figure 1.4 The Structure of Trifluoperazine

forms of cyclic-AMP phosphodiesterase (Weiss, Fertel, Figlin and Uzunov, 1974; Levin and Weiss, 1976), and it was later shown that this inhibition was achieved through the interaction of the drugs with calmodulin rather than phosphodiesterase (Levin and Weiss, 1977, 1979). The binding of TFP to calmodulin was observed to be Ca^{2+}-dependent at low concentrations of drug and enhancement of 10 to 100-fold was obtained in the presence of 100 μM Ca^{2+} (Levin and Weiss, 1977). Trifluoperazine concentrations of the order of 100 μM, however, led to Ca^{2+}-independent binding (Levin and Weiss, 1978). The Ca^{2+}-dependent binding of TFP to calmodulin appeared to involve two high affinity sites (Kd = 1-1.5 μM) while Ca^{2+}-independent binding involved a large number of low affinity sites (Kd = 5 mM) (Levin and Weiss, 1977, 1978). Other divalent cations which substituted for Ca^{2+} were $Sr^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$, while Ba^{2+} and Mg^{2+} were ineffective (Levin and Weiss, 1977).

Levin and Weiss (1978) have described the selectivity of low concentrations of TFP for calmodulins isolated from various sources and a series of other Ca^{2+} binding proteins including myosin light chain, phospholipases A and B, S-100 protein and troponin C. They observed a similar amount of TFP binding to all the calmodulins tested. Among the other Ca^{2+} binding proteins only troponin C bound TFP to any significant extent (approximately one tenth of the amount of TFP bound to calmodulin). At concentrations of TFP above 10 μM or at a pH above 8 the extent of TFP binding was similar for both troponin C and calmodulin.

The structural specificity of the phenothiazine-calmodulin interaction was investigated by Roufogalis (1981). Four analogues of chlorpromazine, differing in the position of chlorine substitution on the aromatic ring were almost equally potent in antagonizing calmodulin activation, while only the two-chloro analogue possessed tranquilizer activity. Roufogalis concluded that the phenothiazine-calmodulin interaction was structurally non-specific. Norman, Drummond and Moser (1979) have reported that the property of calmodulin antagonists which correlated best with their potency as inhibitors was their octanol/water partition coefficients (degree of hydrophobicity). This observation is consistent with the findings of La Porte, Wierman and Storm (1980) that the binding of Ca^{2+} to calmodulin brought about the exposure of a hydrophobic region on this protein. Presumably this is the site of calmodulin binding to its effector enzymes and also the site of Ca^{2+}-dependent antagonism by drugs.

Due to the hydrophobicity of the calmodulin antagonists, these drugs may have other sites of action besides calmodulin itself. The most important of these may be their partitioning into membrane lipid and interaction with the hydrophobic domains of membrane proteins (Corps, Hesketh and Metcalf, 1982; Guth and Spirtes, 1964; Frisk-Holmberg and Kleijn, 1972; Seeman, 1972). Recently a number of reports have shown direct effects of calmodulin antagonists on enzyme activity in the absence of calmodulin. Vincenzi, Adunyah, Niggli and Carafoli (1982) observed that TFP as well as a number of other calmodulin antagonists were inhibitory to the red blood cell plasma membrane ATPase reconstituted into lipid vesicles both in the presence and absence of

calmodulin. Louis, Turnquist and Jarvis (1983) have shown an inhibition of calmodulin-dependent and calmodulin-independent cardiac SR functions by R24571. Schatzman, Wise and Kuo (1981) suggested that TFP and other calmodulin antagonists were interacting with phospholipid to produce inhibition of a phospholipid-sensitive Ca^{2+}-dependent protein kinase.

Perhaps some of the disadvantages of the currently available calmodulin antagonists may be overcome, at least in sub-cellular systems, by the use of a new form of antagonist. Newton and Klee (1984) have reported the use of CAPP-calmodulin, a calmodulin molecule with a phenothiazine molecule (norchlorpromazine) conjugated to it, as a calmodulin antagonist. They observed potent competitive antagonist activity for phosphodiesterase and myosin light chain kinase. The inherent selectivity of the inactive CAPP-calmodulin conjugate for calmodulin binding sites may lessen many current problems of specificity.

1.12 Pharmacological Findings in Malignant Hyperpyrexia Susceptible Muscle

Muscle biopsy specimens from MHS individuals and MHS swine display abnormal contracture responses to a variety of chemical stimuli. Halothane and succinylcholine (ANECTINE; Wellcome Australia Ltd) caused an increased contracture in MH muscle *in vitro* when compared with control muscle (Ellis, Keaney, Harriman, Sumner, Kyei-Mensah, Tyrrell, Hargreaves, Parikh and Mulrooney, 1972; Moulds and Denborough, 1974b, 1974c; Halsall and Ellis, 1979). Contractures induced by caffeine (Kalow, Britt, Terreau and Haist, 1970) and

thymol (the preservative used in the commercial halothane preparation) (Okumura, Crocker and Denborough, 1979) were also increased in MH muscle, as was the contracture produced by potassium chloride depolarization of the muscle cell membrane.

The increased contracture responses in MH skeletal muscle were temperature-dependent (Kalow, Britt, Terreau and Haist, 1970; Nelson, Austin and Denborough, 1977; Sullivan and Denborough, 1981). The optimal temperature for producing drug-induced contractures was 37°C. At 22°C the drug-induced contractures were significantly reduced. Both halothane and caffeine potentiated isometric twitch responses in normal human muscle (Nelson and Denborough, 1977). The muscle relaxant dantrolene sodium prevented and reversed these drug-induced contractures (Austin and Denborough, 1977). These results suggested that drugs which produce abnormal contracture responses in MH muscle were causing an increase in the myoplasmic Ca^{2+} concentration. Dantrolene sodium was able to reverse the abnormal contractures by decreasing the myoplasmic Ca^{2+} concentration (Britt, 1983; Denborough, 1980; Desmedt and Hainaut, 1977).

Attempts have been made to identify the site of the abnormality in MH porcine muscle by treatments which inhibit E-C coupling. Okumura, Crocker and Denborough (1980) found that glycerine treatment of MH muscle, which physically disrupts the t-tubule system, inhibited the abnormal drug-induced contractures to halothane, caffeine, succinylcholine and potassium chloride. These authors also found that pretreatment of MH muscle with deuterium oxide, which inhibits E-C coupling, inhibited the abnormal contracture responses of MH

muscle. These results suggested that the site of the abnormality in MH muscle lies in the E-C coupling mechanism. Another investigation in porcine MH muscle utilized the Ca^{2+} antagonist 8-(N,N-diethylamino)-octyl-3, 4, 5, -trimethoxybenzoate (TMB-8) (Sullivan, Galloway and Denborough, 1983). Low concentrations of TMB-8 inhibited potassium chloride-induced contractures but potentiated contractures induced by halothane, caffeine and succinylcholine. Higher concentrations of TMB-8 contracted MHS muscle but had little effect on normal muscle. TMB-8-induced hyper-reactivity in MHS muscle could be abolished by glycerinization or by dantrolene sodium. Since TMB-8 had no effect on Ca^{2+} levels in actively loaded MH muscle microsome preparations, the E-C coupling mechanism was again implicated as a probable site of the MH abnormality.

Nelson and Schochet (1982) have proposed that MH may be a disease of specific myofibre type. They have shown that a porcine MH muscle with a high proportion of type I fibres (trapezius) has a greater abnormal contracture response to halothane than one with a low proportion of type I fibres (gracilis).

1.13 Biochemical Findings in Malignant Hyperpyrexia

1.13.1 Skeletal Muscle

1.13.1.1 The Sarcoplasmic Reticulum

The skeletal muscle SR is the organelle which is responsible for the uptake and storage of myoplasmic Ca^{2+}, and for the release of stored Ca^{2+} to initiate contraction and muscle cell activation. Because of its central role in muscle cell Ca^{2+} metabolism a number of studies have investigated the function of the SR in MH muscle using isolated

membrane preparations. Studies of Ca^{2+} uptake by human MH muscle SR preparations have been equivocal (Table 1.3). A reduction in the Ca^{2+} uptake rate by MHS SR preparations compared with control SR preparations has been reported (Britt, Kalow, Gordon, Humphrey and Rewcastle, 1973; Isaacs and Heffron, 1975). However, in other studies no difference between Ca^{2+} uptake in MHS and control preparations has been found (Kalow, Britt, Terreau and Haist, 1970; Dhalla, Sulakhe, Clinch, Wade and Naimark, 1972; Blanck, Gruener, Suffecool and Thompson, 1981). Studies of the effect of halothane on Ca^{2+} uptake by MHS and control SR preparations have also produced mixed results. In the presence of halothane, Ca^{2+} uptake by MHS and control SR preparations was unaffected (Isaacs and Heffron, 1975), inhibited to a similar degree (Blanck, Gruener, Suffecool and Thompson, 1981), MHS Ca^{2+} uptake inhibited while control was not (Britt, Kalow, Gordon, Humphrey and Rewcastle, 1973; Kalow, Britt, Terreau and Haist, 1970) and control inhibited more than MHS (Dhalla, Sulakhe, Clinch, Wade and Naimark, 1972).

Studies of porcine MHS SR Ca^{2+} uptake have led to no firm conclusions (Table 1.3). Ca^{2+} uptake has been reported to be increased in MHS SR preparations when compared with controls (Berman and Kench, 1973; Brucker, Williams, Popinigis, Galvez, Vail and Taylor, 1973; Britt, Endrenyi and Cadman, 1975), normal (Nelson, Jones, Venable and Kerr, 1972; Denborough, Hird, King, Marginson, Mitchelson, Naylor, Rex, Zapf and Condron, 1973; White, Collins and Denborough, 1983) or decreased when compared with controls (McIntosh, Berman and Kench, 1977; Nelson, 1978; Gronert, Heffron and Taylor, 1979). The presence of halothane has been reported to increase (Nelson, Jones,

Table 1.3 Ca^{2+} Uptake by Malignant Hyperpyrexia Susceptible Sarcoplasmic Reticulum Preparations in Relation to Control Preparations

Human	
reduced	Britt, Kalow, Gordon, Humphrey and Rewcastle (1973)
reduced	Isaacs and Heffron (1975)
similar	Kalow, Britt, Terreau and Haist (1970)
similar	Dhalla, Sulakhe, Clinch, Wade and Naimark (1972)
similar	Blanck, Gruener, Suffecool and Thompson (1981).
Porcine	
reduced	McIntosh, Berman and Kench (1977)
reduced	Nelson (1978)
reduced	Gronert, Heffron and Taylor (1979)
similar	Nelson, Jones, Venable and Kerr (1972)
similar	Denborough, Hird, King, Marginson, Mitchelson, Naylor, Rex, Zapf and Condron (1973)
similar	White, Collins and Denborough (1983)
increased	Berman and Kench (1973)
increased	Britt, Endrenyi and Cadman (1975)
increased	Brucker, Williams, Popinigis, Galvez, Vail and Taylor (1973)

Venable and Kerr, 1972) and to decrease (Gronert Heffron and Taylor, 1979) Ca^{2+} uptake by SR preparations from control and MHS porcine muscle. In another study MH SR preparations were sensitive to halothane inhibition of Ca^{2+} uptake whereas control SR preparations were not (Brucker, Williams, Popinigis, Galvez, Vail and Taylor, 1973). The rates of passive Ca^{2+} efflux were similar in MH and control SR preparations (White, Collins and Denborough, 1983). Two recent studies showed abnormalities of Ca^{2+}-induced Ca^{2+} release from MH SR preparations. Nelson (1983) and Ohnishi, Taylor and Gronert (1983) observed that the average Ca^{2+} concentration threshold for Ca^{2+}-induced Ca^{2+} release was markedly lower for MH heavy SR when compared with controls.

Studies on the effects of dantrolene sodium on SR function in porcine MH SR preparations showed no effect on Ca^{2+} uptake (White, Collins and Denborough, 1983) but an inhibitory effect on halothane-induced calcium release (Ohnishi, Taylor and Gronert, 1983).

The phospholipid content of the SR of porcine MH muscle has been investigated by Jardon, Barak, Noffsinger, Chapin and Wingard (1980). These authors found a reduction in phosphatidylcholine and phosphatidylethanolamine in the SR of MH muscle when compared to control muscle SR.

1.13.1.2 Muscle Proteins

No specific muscle protein abnormalities have been reported in MHS tissue. Lorkin and Lehmann (1983a) used two dimensional gel electrophoresis to study whole muscle extracts, and EDTA and myosin

light chain extracts of control and MH porcine muscle. They found no obvious differences in the major contractile proteins or glycolytic enzymes. These authors (Lorkin and Lehmann, 1983b) have also studied troponin C (by tryptic peptide mapping) from control and MH porcine skeletal muscle and found no differences.

1.13.1.3 Mitochondria

Cheah and Cheah (1978, 1979) proposed that an abnormality of mitochondrial Ca^{2+} storage may be responsible for porcine MH. They found an increased Ca^{2+} efflux rate in mitochondria isolated from MHS muscle, and considered that the released Ca^{2+} was sufficient to trigger rapid glycolysis, activation of the myofibrillar ATPase and activation of phosphorylase kinase.

1.13.1.4 Electrophysiology

Gallant, Godt and Gronert (1979) have observed that halothane depolarized the sarcolemma of MHS porcine muscle but not control muscle, and that dantrolene reduced or reversed this depolarization. The suggestion by these authors that the basic abnormality in MH involved the sarcolemma is not supported by another investigation where no effect of halothane on sarcolemmal resting membrane potential of MHS muscle was observed (Bradley, Ward, Murchison, Hall and Woolf, 1973).

The electromechanical studies of Nelson, Flewellen and Arnett (1983) showed that the time interval from nerve stimulation to onset of mechanical tension was longer for muscle from MHS pigs than for control muscle both *in vitro* and *in vivo*. The *in vivo* kinetics of

compound muscle action potential formation were similar in control and MHS muscle, but the interval from the peak of the muscle action potential to tension onset was longer in MHS muscle. These authors concluded that these results could have been explained by an abnormality of E-C coupling in MH.

1.13.1.5 Muscle Energy Metabolism

When MH skeletal muscle was exposed to halothane, myoplasmic Ca^{2+} levels rose and actomyosin ATPase was activated. This led to a reduction in the creatine phosphate concentration in MHS muscle much greater than that observed in control muscle. This applied to both human MHS muscle (Britt, Endrenyi, Kalow and Peters, 1967) and porcine MHS muscle (Harrison, Saunders, Biebuyck, Hickman, Dent, Weaver and Terblanche, 1969; Berman and Kench, 1973; Nelson, Jones, Venable and Kerr, 1972). Untreated human MHS muscle (Isaacs, Heffron and Badenhorst, 1975) and porcine MHS muscle (Van den Hende, Lister, Muylle, Doms and Oyaert, 1976) have also been shown to display reduced creatine phosphate levels. The muscle enzyme CPK has been found to be elevated in the serum of many MHS individuals, due to leaky muscle membranes (Britt, 1977).

A deficiency of adenylate kinase, the enzyme which catalyses the conversion of two molecules of ADP to one molecule of ATP and one molecule of AMP, has been reported in human MH (Schmitt, Schmidt and Ritter, 1974). Also, a binding site for halothane on the adenylate kinase molecule was reported by Sachsenheimer, Pai, Schulz and Schirmer (1977). Marjanen, Shaw and Denborough (1983) investigated

the structure and activity of adenylate kinase in control and MH porcine muscle but failed to find any differences.

Muscle phosphorylase, the enzyme which liberates glucose-1-phosphate from glycogen, is present in two forms. The a or active form and the b or inactive form are interconverted by a Ca^{2+}-dependent phosphorylase kinase. Willner, Wood, Cerri and Britt (1980) observed the ratio of phosphorylase a to total phosphorylase in human MHS skeletal muscle to be higher than control values. Such an imbalance might help fuel explosive glycolysis during a MH episode and contribute to lactic acid formation. Traynor, Van Dyke and Gronert (1983), however, found no significant differences between the phosphorylase a to total phosphorylase ratio in the muscle of their group of MHS subjects and controls. Willner (1984) and Gronert and Van Dyke (1984) have discussed these different findings.

1.13.1.6 Cyclic Nucleotides

An increase in the activity of adenylate cyclase and high cyclic-AMP levels have been reported in human MHS skeletal muscle (Willner, Cerri and Wood, 1981). These authors suggested that these changes may result in the observed increase in levels of phosphorylase activation in human MHS muscle through activation of a cyclic-AMP dependent phosphorylase kinase (Willner, Wood, Cerri and Britt, 1980). Willner, Cerri and Wood (1981) also proposed that increased phosphorylation of SR proteins by cyclic-AMP dependent protein kinases may contribute to abnormalities of SR Ca^{2+} transport in MHS muscle. Porcine MHS muscle has also been found to contain cyclic-AMP

concentrations significantly higher than control at 3 minutes post mortem (Ono, Topel and Althen, 1976, 1977).

1.13.2 Other Tissues

1.13.2.1 Platelets

Rosenberg, Fisher, Reed and Adonizio (1981) have studied the aggregation of platelets from MHS individuals. They found no consistent or specific difference in MH platelet aggregation when compared to control platelets. Halothane-induced platelet ATP depletion has also been investigated in human MH. Solomons, McDermott and Mahowald (1980) found a decrease in ATP in MH platelets when compared to control, whereas Giger and Kaplan (1983) found no difference.

1.13.2.2 Pancreas

Since Ca^{2+} is also involved in the stimulus-secretion coupling of insulin release from the pancreatic beta-cell, Denborough, Warne, Moulds, Tse and Martin (1974) studied glucose-stimulated insulin secretion in MHS individuals. These authors observed an increased glucose-induced insulin response in MHS individuals when compared to controls, and concluded that the membrane abnormality of skeletal muscle in MH may be generalized.

1.13.2.3 Catecholamines

Lister, Hall and Lucke (1974) and Gronert and Theye (1976) have observed large increases in circulating catecholamine levels during porcine MH episodes. These increases might be produced by a Ca^{2+}-dependent release of catecholamines from the adrenal medulla

(Douglas and Rubin, 1961) and may contribute to the stimulation of heat production in MH (Denborough, 1980).

1.13.2.4 Erythrocytes

Lorkin and Lehmann (1983b) studied calmodulin of erythrocytes from control and MHS swine by two dimensional electrophoresis and found no differences.

1.4 Summary

Malignant hyperpyrexia is a life-threatening reaction to general anaesthesia which occurs in individuals with an underlying muscle disease. The symptoms of MH include hyperpyrexia and rigidity of the skeletal musculature. The muscle relaxant dantrolene sodium is now available as a specific treatment of MH. The symptomology of MH and the *in vitro* pharmacology of MHS skeletal muscle have suggested that the basic abnormality in MH involves an abnormal regulation of myoplasmic Ca^{2+} levels. Investigations of the Ca^{2+} regulatory functions of MHS skeletal muscle, however, have not shown a consistent or specific abnormality in these functions. A number of studies have implicated the E-C coupling mechanism as the site of the basic abnormality in MHS skeletal muscle.

CHAPTER 2 THE PHARMACOLOGY OF CALMODULIN ANTAGONISTS IN CONTROL AND MALIGNANT HYPERPYREXIA SUSCEPTIBLE PORCINE SKELETAL MUSCLE.

2.1 Introduction

Malignant Hyperpyrexia susceptible skeletal muscle exhibits hypercontractility to 3% halothane, 2 mM caffeine, 1 mM succinylcholine and 80 mM potassium chloride *in vitro* in a pharmacological muscle strip preparation (Okumura, Crocker and Denborough, 1979). This hypercontractility may be explained by an abnormality of Ca^{2+} regulation in MHS muscle leading to a higher than normal myoplasmic Ca^{2+} concentration (Denborough, 1980). Since the Ca^{2+} regulatory protein calmodulin has been implicated in many Ca^{2+}-dependent processes (Chapter 1.11), its possible role in MH was investigated. In this chapter the possible role of calmodulin in producing the skeletal muscle hypercontractility which is characteristic of susceptibility to MH was investigated. This was done by studying the effects of calmodulin antagonist drugs on control and MHS porcine muscle contractility *in vitro*.

Table 2.1 The Calmodulin Antagonist Drugs used in the Present Pharmacological Studies

	Octanol/Water Partition Coefficient (Seeman, 1977)	I_{50} for Calmodulin (μM)	
Penfluridol (PEN)	40,000,000	2.5	Levin and Weiss, 1979
Pimozide (PIM)	2,000,000	7	Levin and Weiss, 1979
Trifluoperazine (TFP)	1,700,000	10	Levin and Weiss, 1979
Fluphenazine (FPZ)	912,000	10	Roufogalis, 1982
Haloperidol (HPD)	555,000	60	Levin and Weiss, 1979
Chlorpromazine (CPZ)	191,000	42	Levin and Weiss, 1979
Promethazine (PRO)	22,400	340	Weiss, Prozialeck and Wallace, 1982

2.2 Materials and Methods

2.2.1 Drugs and Reagents

The following gifts of drugs are gratefully acknowledged: TFP from Smith, Kline and French Laboratories Australia; PRO and CPZ from May and Baker Australia Pty Ltd; FPZ from E.R. Squibb and Sons; HPD from Searle Laboratories; PEN and PIM from Janssen Pharmaceutica, N.V., Belgium; and dantrolene sodium from Norwich Pharmaceuticals.

The drugs used in the pharmacological experiments and diagnostic testing were thymol-free halothane, kindly supplied by ICI Australia, and a pharmaceutical preparation of succinylcholine (ANECTINE, Wellcome Australia Ltd).

All other reagents were of analytical grade.

2.2.2 Experimental Animals

Malignant hyperpyrexia susceptible swine were bred from MHS stock and from crosses of MHS and non-affected stock. Control swine were identified from mixed litters and also obtained commercially. The animals were housed at the Animal Breeding Establishment, Australian National University.

2.2.3 Anaesthesia

The animals were premedicated with 1.5-2 mg/kg of Stresnil (Smith, Kline and French Laboratories Australia Ltd) intramuscularly. Anaesthesia was induced with intravenous thiopentone (Pentothal Sodium, Abbott Hospital Products) at a dose of 6-8 mg/kg. Anaesthesia was maintained with 66% nitrous oxide in oxygen.

2.2.4 Surgical Procedure

Muscle biopsy specimens were removed from the gracilis muscle. The specimens were 4 cm long, 1 cm wide and 0.5 cm thick and were held in clamps to maintain resting tension. Immediately after excision the specimens were placed into Ringer solution at 37^{o}C which was bubbled with 5% carbon dioxide in oxygen (carbogen). The composition of the Ringer solution was 121 mM NaCl, 5.4 mM K Cl, 3.5 mM $CaCl_2$, 1.2 mM Mg SO_4, 1.2 mM NaH_2 PO_4, 15 mM $NaHCO_3$ and 11.5 mM glucose (pH 7.3).

2.2.5 The Organ Bath Preparation

Muscle fibre bundles of approximately 2 mm diameter were dissected from the biopsy specimen and small metal rings were attached to both ends of the bundle. This preparation was then suspended vertically in the organ bath (Figure 2.1). The muscle strip was attached at one end to a fixed glass rod and at the other to a Watson Victor force transducer (F.0202). When lowered into the organ bath, the muscle strip was bathed in 25 ml of Ringer solution continuously bubbled with carbogen. Signals from the force transducer were recorded using a Perkin-Elmer 56 chart recorder after amplification by a San-EI 6M51 Strain Amplifier. After setting the initial resting tension for each muscle strip at approximately 1 g, the viability of the preparation was confirmed by 30V electrical stimuli.

Stock solutions of drugs and chemicals were: caffeine, 100 mM in Ringer; potassium chloride, 4M in Ringer; TFP, FPZ and PRO, 25 mM in Ringer; CPZ 25 mM in distilled water; HPD, PEN and PIM, 25 mM in dimethylsulfoxide (DMSO); and dantrolene sodium, 15 mM in DMSO. Thymol-free halothane was administered by passing the carbogen through a Dragewick vapourizer.

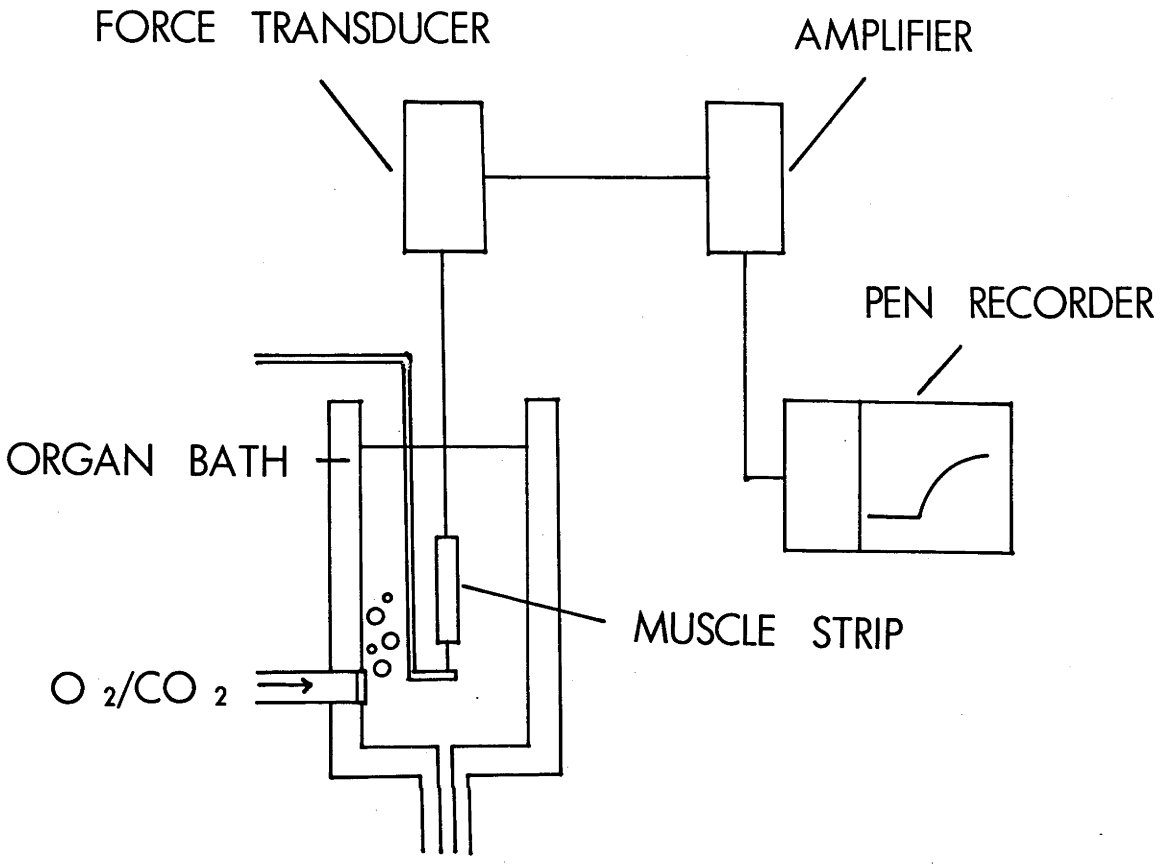


Figure 2.1 Diagram of the Organ Bath and Associated Equipment.

2.2.6 Data Storage and Analysis

Pharmacological tension responses were encoded and stored in a computer-based "Statistical Package for the Social Sciences" (Nie, Hull, Jenkins, Steinbrenner and Bent, 1970). The data was analysed using Student's t-test with both pooled and separate variance estimates.

The mean contracture figures for halothane, caffeine, succinylcholine and potassium chloride of control and MHS muscle include diagnostic testing of pigs between April 1981 and April 1984.

Where exposure of the muscle to halothane, caffeine, succinylcholine or potassium chloride was preceded by treatment with a calmodulin antagonist, any contracture due to the calmodulin antagonist was not included in the subsequent measurement.

2.3 Results

2.3.1 Identification of Control and Malignant Hyperpyrexia Susceptible Swine

The identification of control and MHS swine was carried out *in vitro* using a muscle biopsy specimen from the gracilis muscle. Fibre bundles from this specimen were suspended in an organ bath (Figure 2.1) and their contracture responses to 3% halothane, 2 mM caffeine, 1 mM succinylcholine and 80 mM potassium chloride were recorded. Muscle strips from MHS swine displayed a characteristic hypercontractility to these agents whereas those from control swine did not (Figure 2.2, Table 2.2).

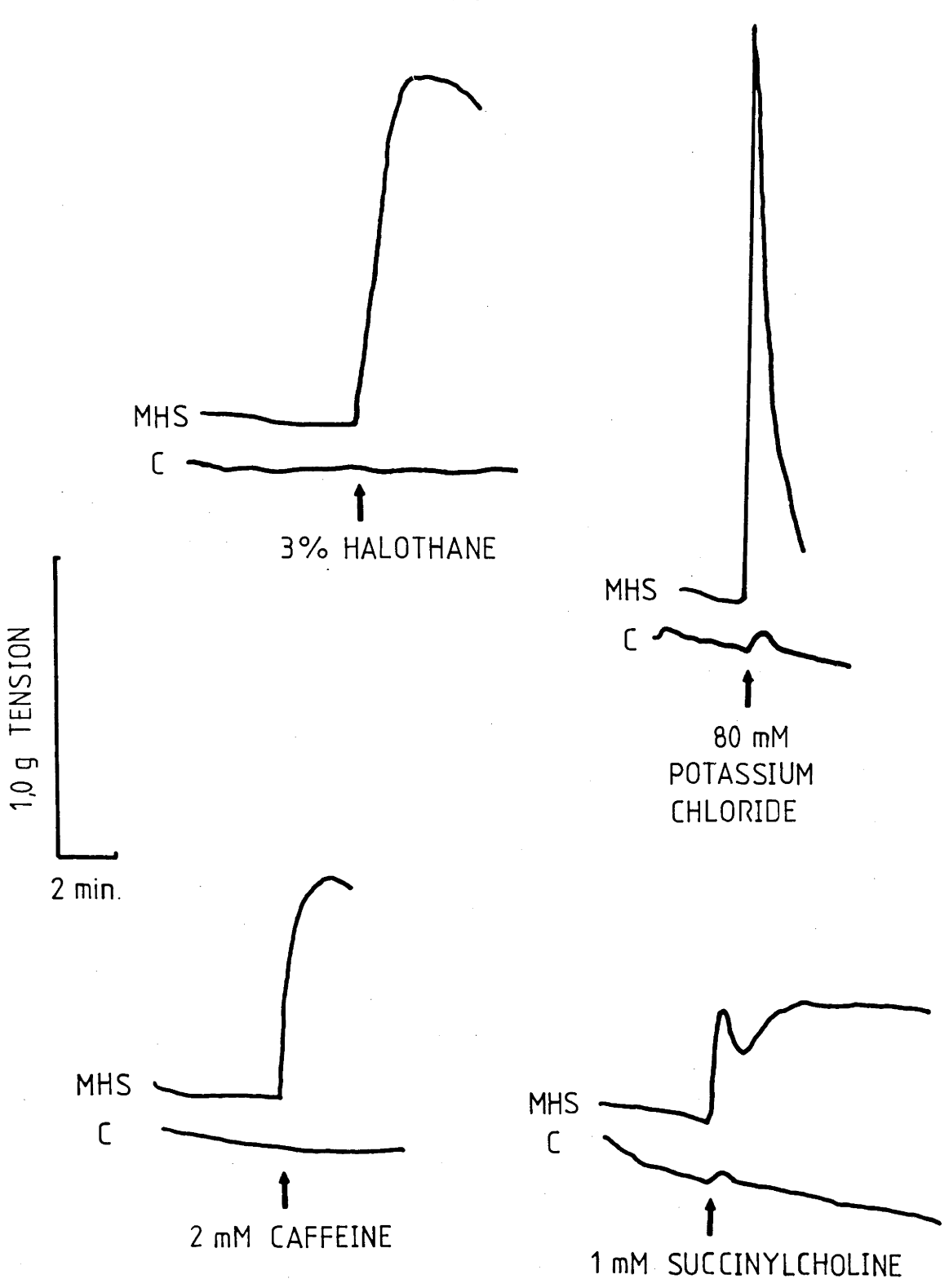


Figure 2.2 Typical Contracture Responses of Control and Malignant Hyperpyrexia Susceptible Muscle to Halothane, Caffeine, Succinylcholine and Potassium Chloride.

Table 2.2 **Contracture Responses of Control and Malignant Hyperpyrexia Susceptible Swine Skeletal Muscle when exposed to Halothane, Caffeine, Succinylcholine and Potassium Chloride.**

	g tension (mean ± S.E.)		P value
	control	MHS	
Halothane 3%	0.0284 ± 0.006 (93)	0.7567 ± 0.045 (187)	<0.001
Caffeine 2 mM	0.0359 ± 0.01 (90)	0.5317 ± 0.036 (149)	<0.001
Succinylcholine 1 mM	0.0701 ± 0.021 (79)	0.3724 ± 0.029 (125)	<0.001
Potassium Chloride 80 mM	0.1264 ± 0.06 (80)	1.1513 ± 0.087 (119)	<0.001

2.3.2 Contracture Responses Induced by Calmodulin Antagonist Drugs

The phenothiazine calmodulin antagonists TFP, CPZ, FPZ and PRO, and the butyrophenone HPD induced contractures in both control and MHS skeletal muscle strips at μM concentrations when added directly to the organ bath (Figures 2.3a, 2.4, 2.5, 2.6, 2.7, 2.8). Promethazine was the least active of this group of drugs in inducing muscle contracture and required an organ bath concentration of at least 200 μM before contracture was observed in control or MHS muscle (Figure 2.7). Contracture was induced by TFP (Figure 2.4), CPZ (Figure 2.5), FPZ (Figure 2.6) and HPD (Figue 2.8) at an organ bath concentration of 50 μM in both control and MHS muscle. The size of the contractures induced by 50 μM TFP, 50 μM CPZ and 50 μM HPD were significantly larger in MHS muscle when compared with controls (Figure 2.9).

The calmodulin antagonists PEN and PIM did not induce contracture of either control or MHS muscle strips. Since these two drugs were the most insoluble used (Table 2.1), the aqueous organ bath environment was slightly modified to facilitate delivery of these drugs to the muscle strip. To facilitate the solubility of these drugs the Ringer solution was prepared without SO_4^{2-} or with up to 5% DMSO. Since almost all of the serum concentration of neuroleptic drugs such as PEN and PIM is bound to serum proteins (Seeman, 1977), up to 0.5% of bovine serum albumin was added to the Ringer solution. None of these modifications were successful in inducing PEN or PIM contractures in control or MHS muscle strips.

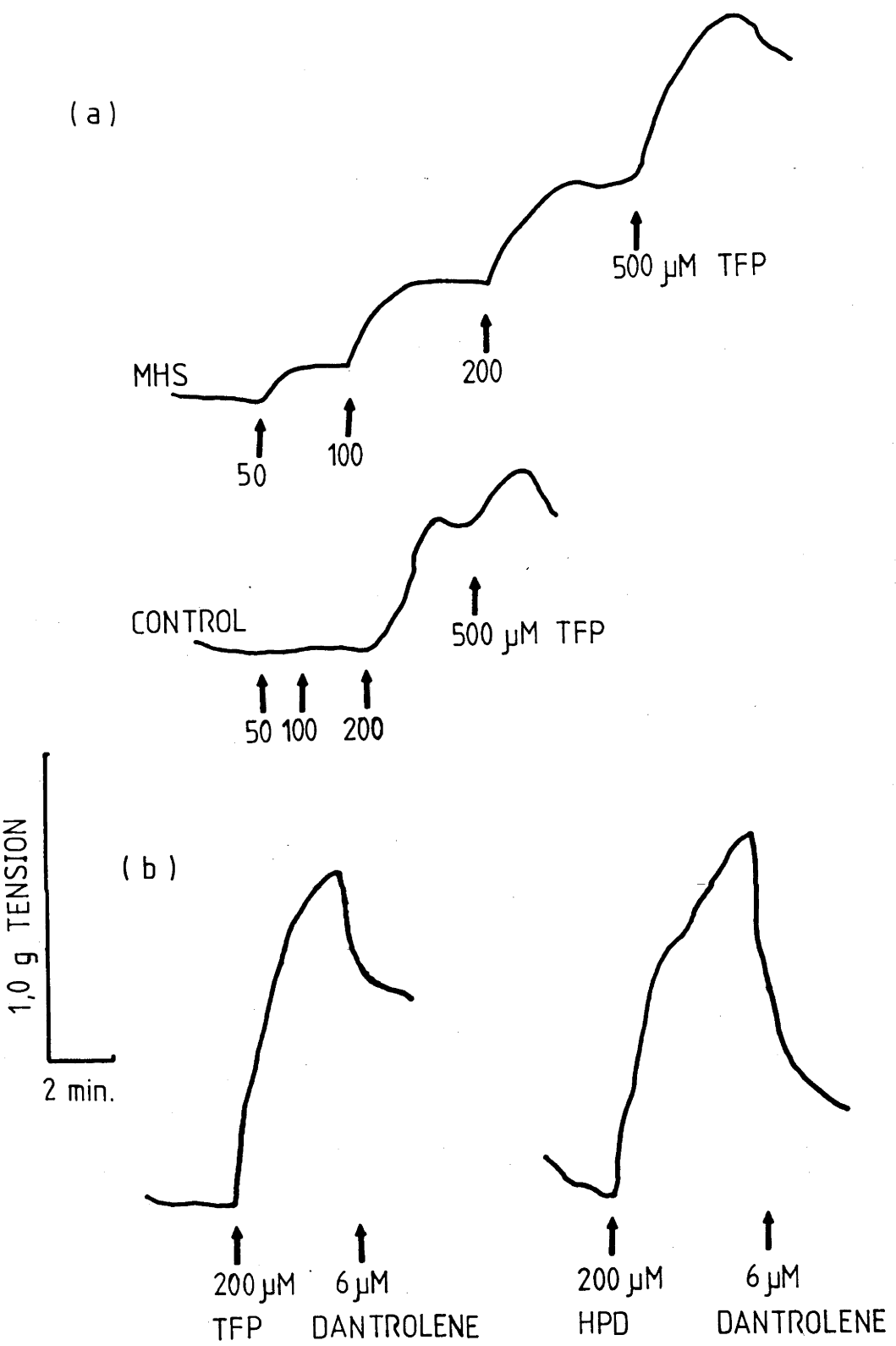


Figure 2.3 The Effect of Trifluoperazine on Control and Malignant Hyperpyrexia Susceptible Muscle. (a) Typical Dose Responses to Trifluoperazine. (b) Partial Reversal of Trifluoperazine Contracture compared to Reversal of Haloperidol Contracture in Control Muscle.

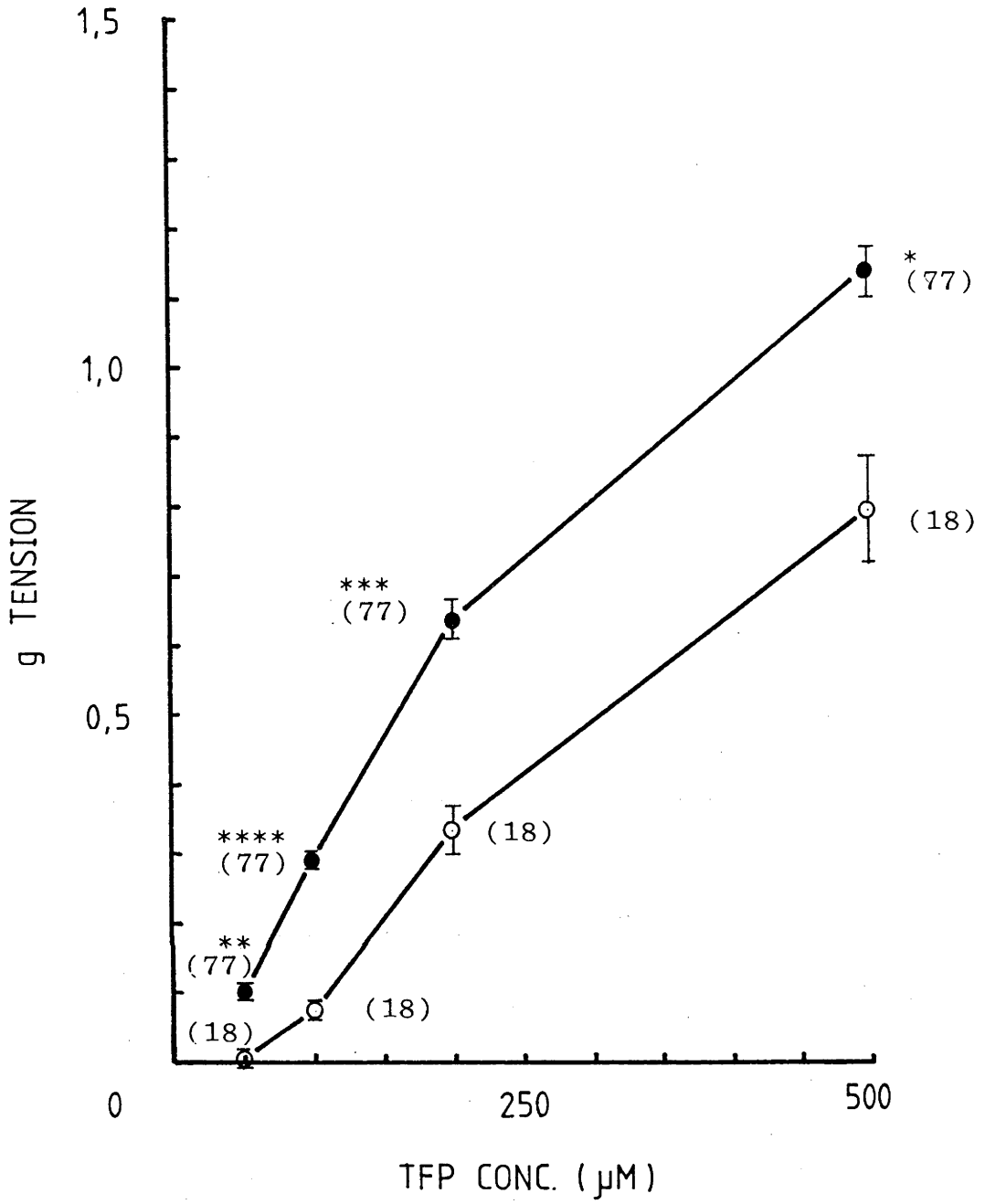


Figure 2.4 Dose Response of Contracture Induced by Trifluoperazine in Control (o) and Malignant Hyperpyrexia Susceptible (•) Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

* Significanly greater than control $p < 0.05$

** " " " " $p < 0.01$

*** " " " " $p < 0.005$

**** " " " " $p < 0.001$

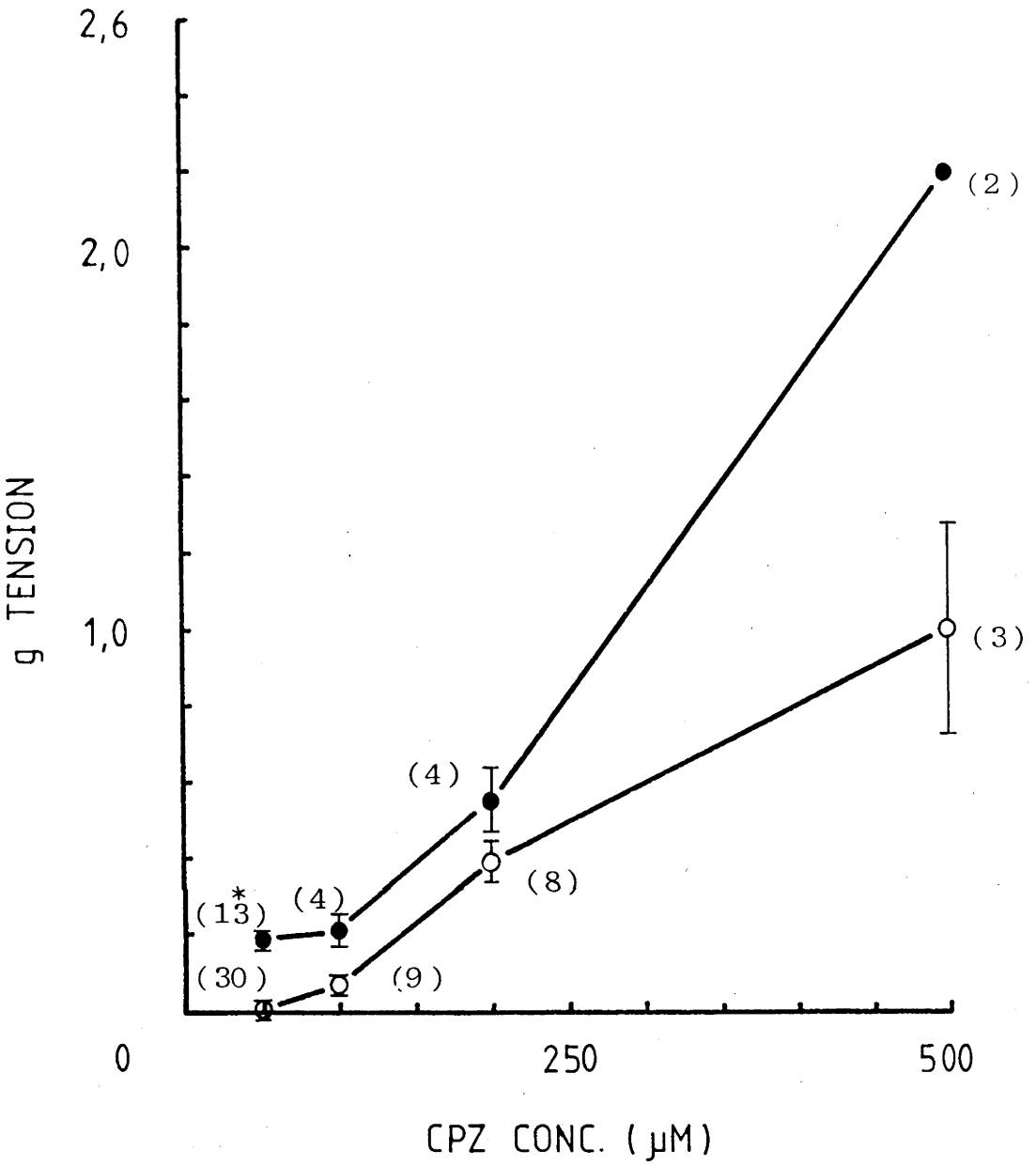


Figure 2.5 Dose Response of Contracture Induced by Chlorpromazine in Control (o) and Malignant Hyperpyrexia Susceptible (•) Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

* Significantly greater than control $p < 0.02$

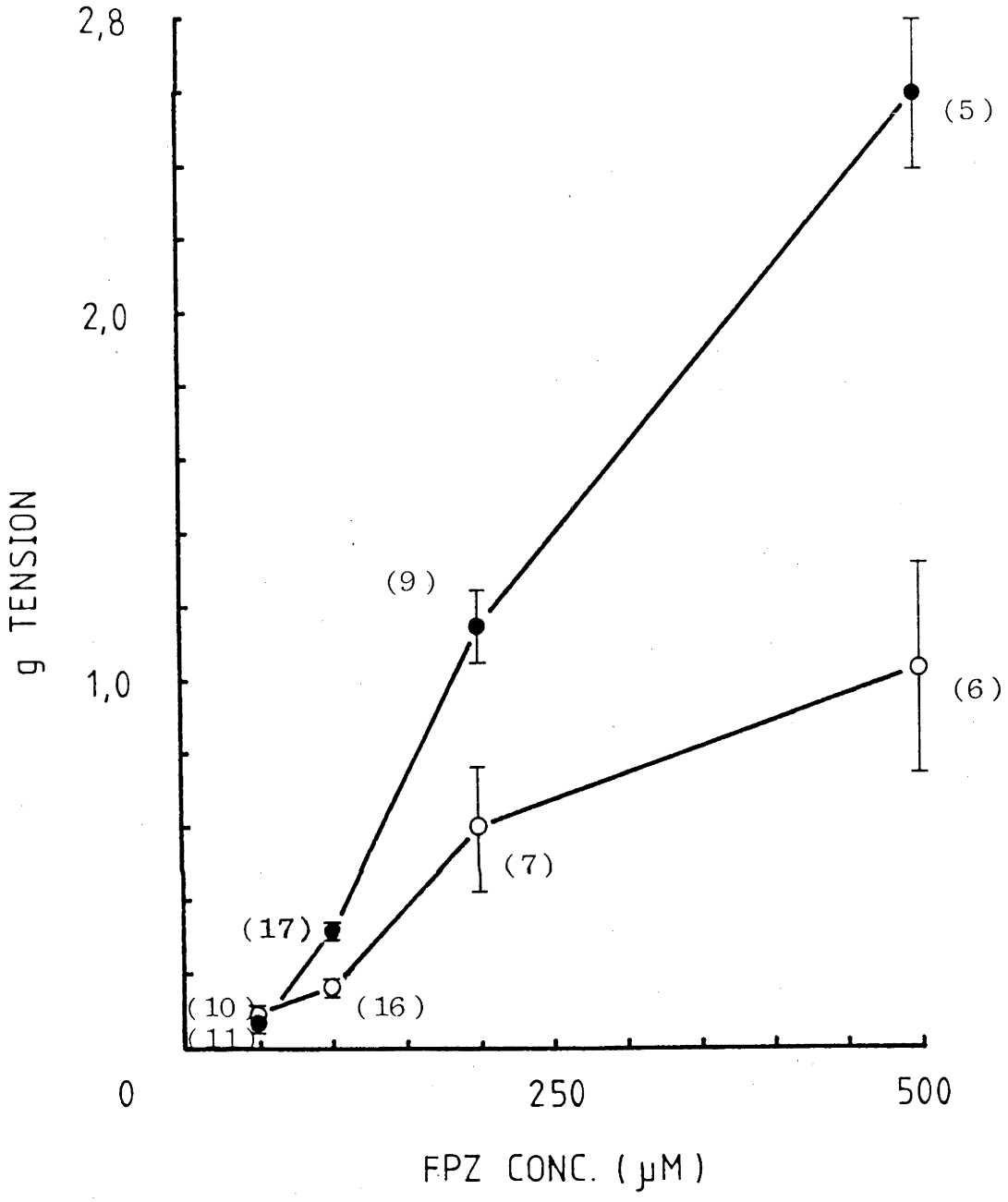


Figure 2.6 Dose Response of Contracture Induced by Fluphenazine in Control (o) and Malignant Hyperpyrexia Susceptible (•) Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets. None of the MHS values are significantly different from control values.

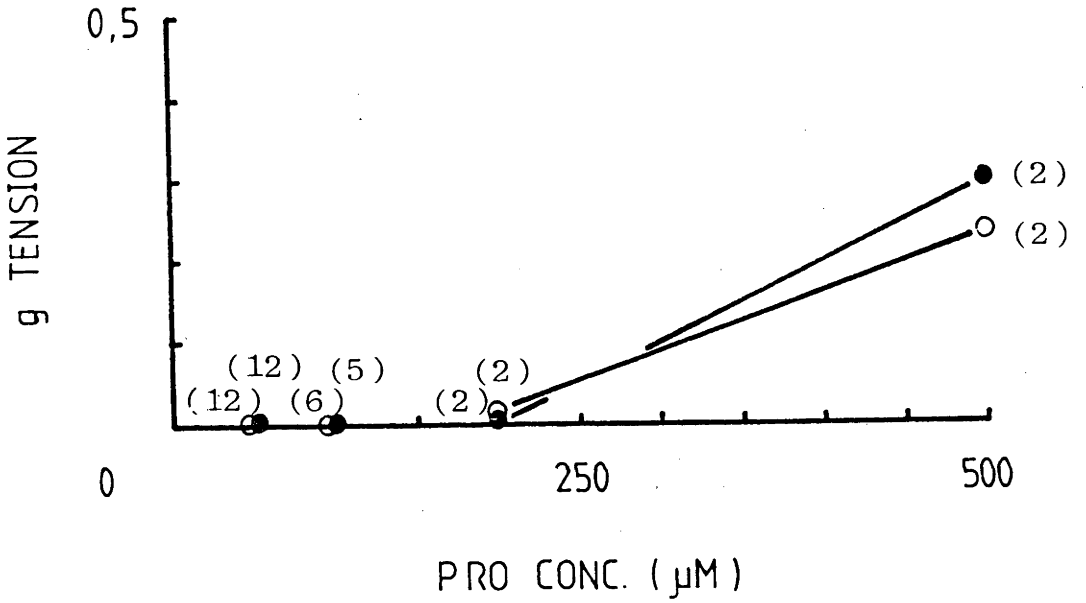


Figure 2.7 Dose Response of Contracture Induced by Promethazine in Control (o) and Malignant Hyperpyrexia Susceptible (•) Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets. None of the MHS values are significantly different to control values.

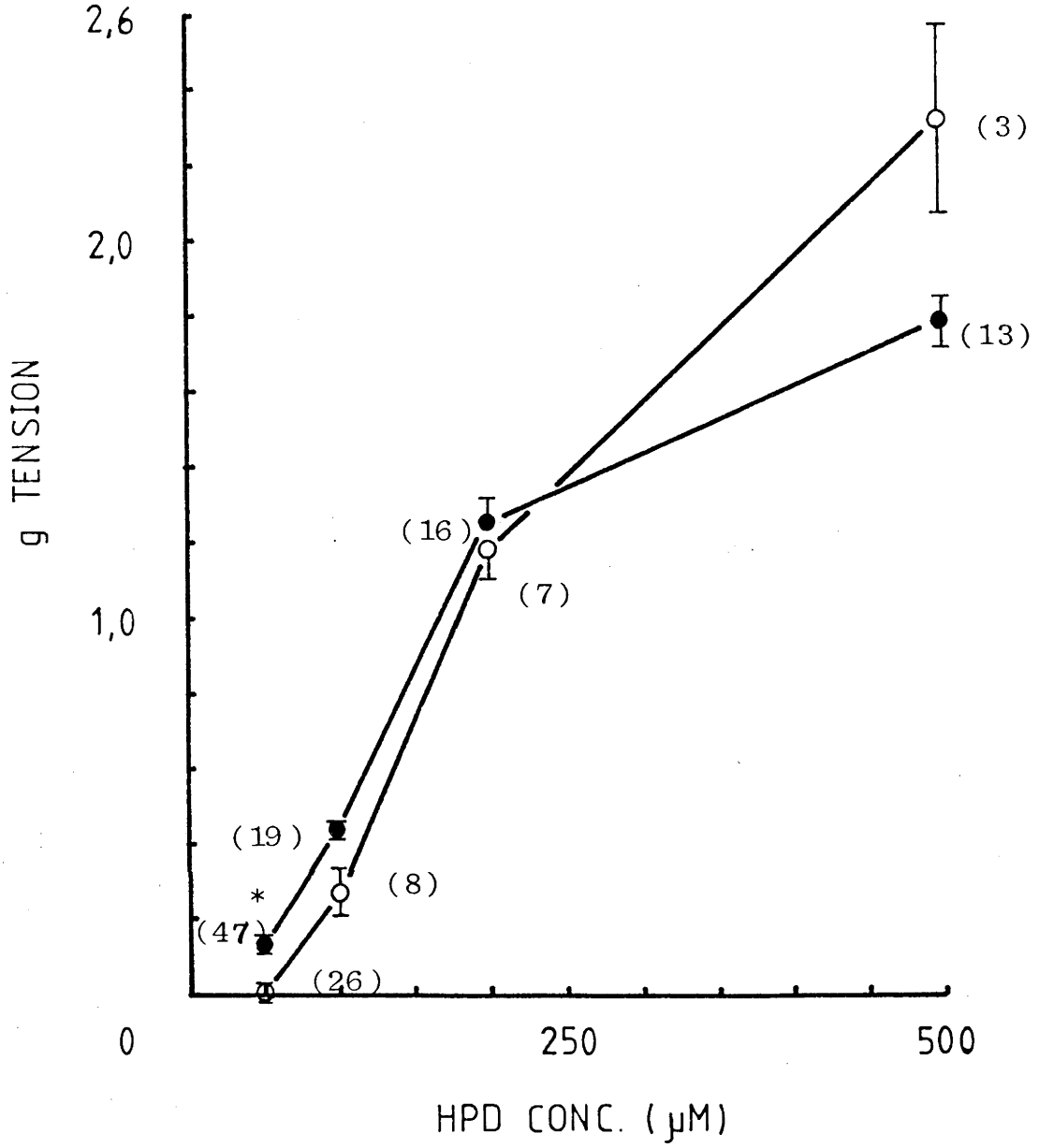


Figure 2.8 Dose Response of Contracture Induced by Haloperidol in Control (o) and Malignant Hyperpyrexia Susceptible (•) Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

* Significantly greater than control $p < 0.001$

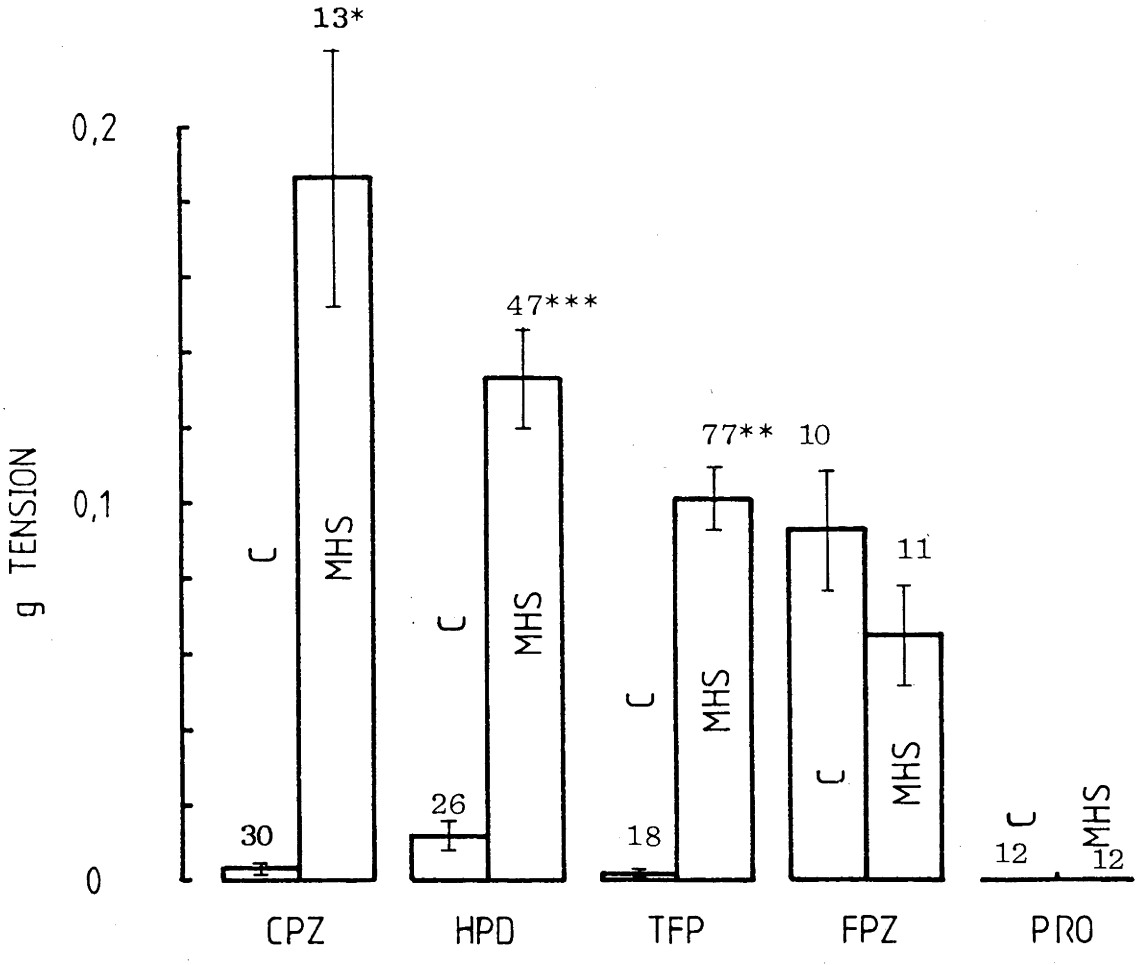


Figure 2.9 Comparison of Contracture Responses Produced by Chlorpromazine, Haloperidol, Trifluoperazine, Fluphenazine and Promethazine at a concentration of 50 μm in Control and Malignant Hyperpyrexia Susceptible Muscle. The results shown are mean values ± S.E.

* Significantly greater than control $p < 0.02$

** " " " " $p < 0.01$

*** " " " " $p < 0.001$

There are no significant differences between contractures produced by chlorpromazine, haloperidol, trifluoperazine and fluphenazine in control or malignant hyperpyrexia susceptible muscle.

2.3.3 The Effects of Dantrolene on Contractures Induced by Calmodulin Antagonists

Dantrolene sodium was used in the organ bath experiments at a concentration of 6 μM (Denborough, 1980). Dantrolene sodium did not completely reverse the contractures caused by calmodulin antagonists in control and MHS muscle strips (Figure 2.3b). The degree of reversal and inhibition of these contractures was greater with HPD than with TFP (Table 2.3). Mean dantrolene sodium reversals of TFP contractures in control and MHS muscle were 14.5% and 29%, respectively. Mean dantrolene sodium reversals of HPD contractures in control and MHS muscle were 78% and 79%, respectively, and were significantly different to the TFP results. The mean dantrolene sodium inhibition of TFP contractures in control and MHS muscle were 67% and 42%, respectively, while dantrolene sodium inhibitions of HPD contractures in control and MHS muscle were 99% and 97%, respectively.

2.3.4 The Effect of Calmodulin Antagonist Drugs on Contractures Induced by Halothane, Caffeine, Succinylcholine and Potassium Chloride in Control Porcine Muscle

The addition of TFP, HPD and CPZ to the organ bath preparation in μM concentrations caused control muscle to behave in a manner similar to MHS muscle. Pretreatment with these drugs for 2 to 5 minutes induced control muscle strips to give contracture responses to 3% halothane, 2 mM caffeine and 1 mM succinylcholine. The responses of control muscle to 80 mM potassium chloride were unaffected by calmodulin antagonist drugs (Figure 2.10). The calmodulin antagonists PEN and PIM did not induce hypercontractility in control muscle.

Table 2.3 A Comparison of the Extent of Dantrolene Sodium Reversal and Inhibition of Trifluoperazine and Haloperidol Contractures in Control and Malignant Hyperpyrexia Susceptible Swine Muscle

	Dantrolene Reversal % (Mean ± SE)		Dantrolene Inhibition % (Mean ± SE)	
	Control	MHS	Control	MHS
Trifluoperazine	14.5 ± 3.4 (10)	29 ± 6.3 (6)	67 ± 6 (6)	42 ± 14.5 (4)
Haloperidol	78 ± 7.4 (5)	79 ± 10.5 (3)	99 ± 0.7 (3)	97 ± 1.2 (3)
P value	<0.001	<0.01	<0.01	<0.05

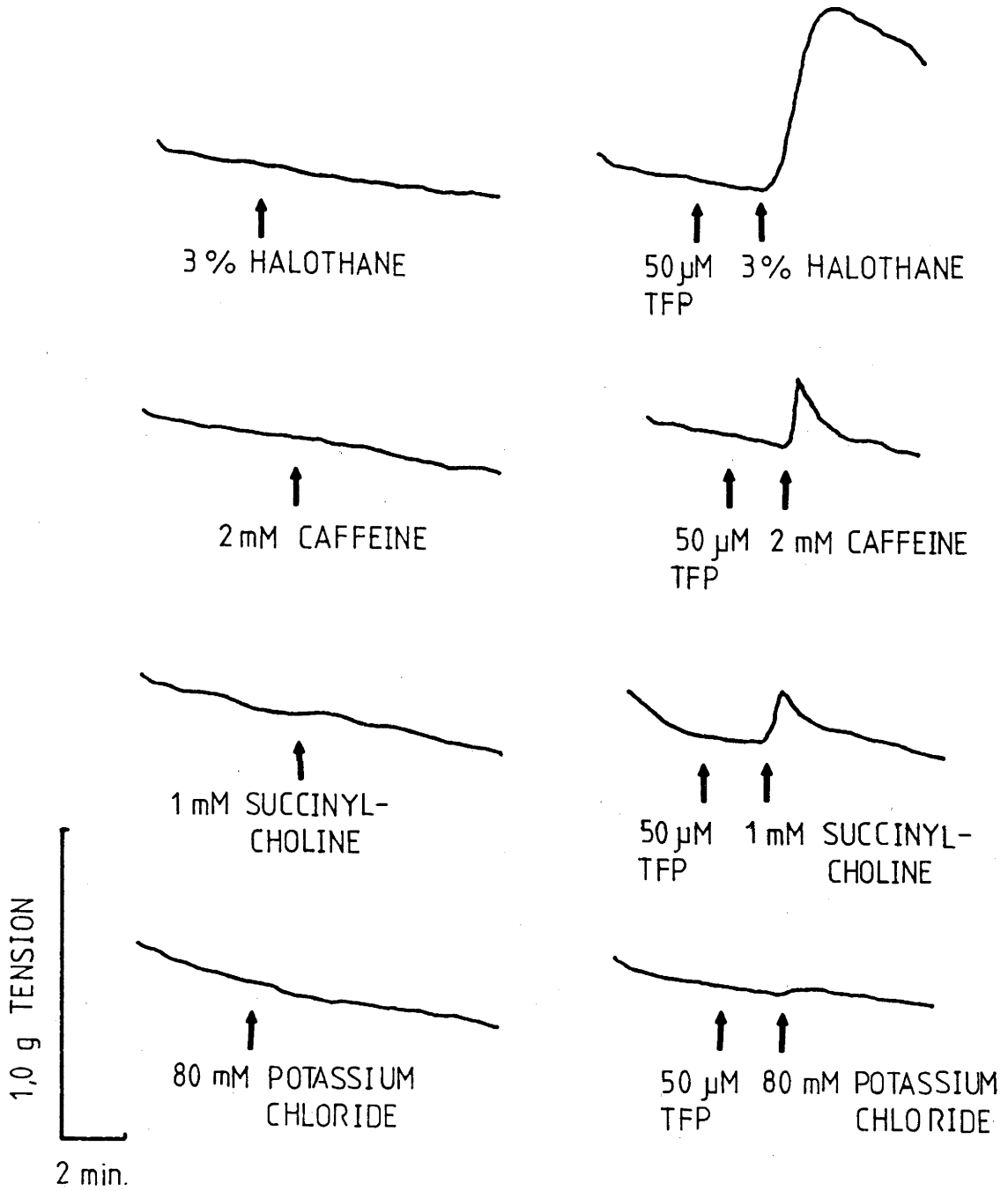


Figure 2.10 Induction of Hypercontractility to Halothane, Caffeine and Succinylcholine by Trifluoperazine in Control Porcine Muscle.

At a concentration of 50 μM, TFP caused control muscle to respond to 3% halothane with a mean contracture of 0.355g, significantly larger than untreated control tissue but significantly less than the mean MHS muscle halothane contracture (Table 2.4). Higher concentrations of TFP pretreatment induced larger halothane contractures, and at 100 μM and 200 μM TFP these halothane contractures were not significantly different from the mean MHS muscle halothane contracture (Figure 2.11). Trifluoperazine-induced caffeine contractures followed the same trend as the TFP-induced halothane contractures. At a concentration of 50 μM TFP a mean caffeine contracture of 0.125 g was recorded, significantly larger than untreated control tissue but significantly less than the mean MHS muscle caffeine contracture (Table 2.4). At concentrations of 100 μM and 200 μM TFP the caffeine contractures were not significantly different from the mean MHS muscle caffeine contracture (Figure 2.12).

The mean TFP-induced succinylcholine contractures in control muscle at TFP concentrations of 50 μM (0.319 g) and 100 μM (0.55 g) were not significantly different from the mean MHS muscle succinylcholine contracture (Figure 2.13). At a TFP concentration of 200 μM the mean succinylcholine contracture in control muscle (0.8 g) was significantly greater than the mean MHS muscle succinylcholine contracture (Figure 2.13).

The mean potassium chloride contractures in control muscle pretreated with 50 μM, 100 μM or 200 μM TFP were not significantly different from the mean untreated control muscle potassium chloride contracture (Table 2.4 and Figure 2.14).

Table 2.4 Hypercontractility Induced in Control Muscle and Potentiation Induced in Malignant Hyperpyrexia Susceptible Muscle by 50 μM Trifluoperazine. The number of experiments are shown in brackets.

Mean Tension (g) ± S.E.

	CONTROL	CONTROL + 50 μM TFP	MHS	MHS + 50 μM TFP
HALOTHANE	0.0284 ± 0.006 (93)	*+ 0.355 ± 0.08 (13)	** 0.7567 ± 0.045 (187)	**# 1.6567 ± 0.24 (9)
CAFFEINE	0.0359 ± 0.01 (90)	*++ 0.1255 ± 0.031 (11)	** 0.5317 ± 0.036 (149)	**# 0.9674 ± 0.158 (17)
SUCCINYLCHOLINE	0.0701 ± 0.021 (79)	** 0.3199 ± 0.082 (9)	** 0.3724 ± 0.029 (125)	**# 0.9150 ± 0.178 (10)
POTASSIUM CHLORIDE	0.1264 ± 0.06 (80)	+++ 0.075 ± 0.037 (10)	** 1.1513 ± 0.087 (119)	** 0.995 ± 0.231 (10)

* Significantly greater than control muscle response (without trifluoperazine) $p < 0.005$

** Significantly greater than control muscle response (without trifluoperazine) $p < 0.001$

\# Significantly greater than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.001$

\+ Significantly less than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.05$

++ Significantly less than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.005$

+++ Significantly less than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.001$

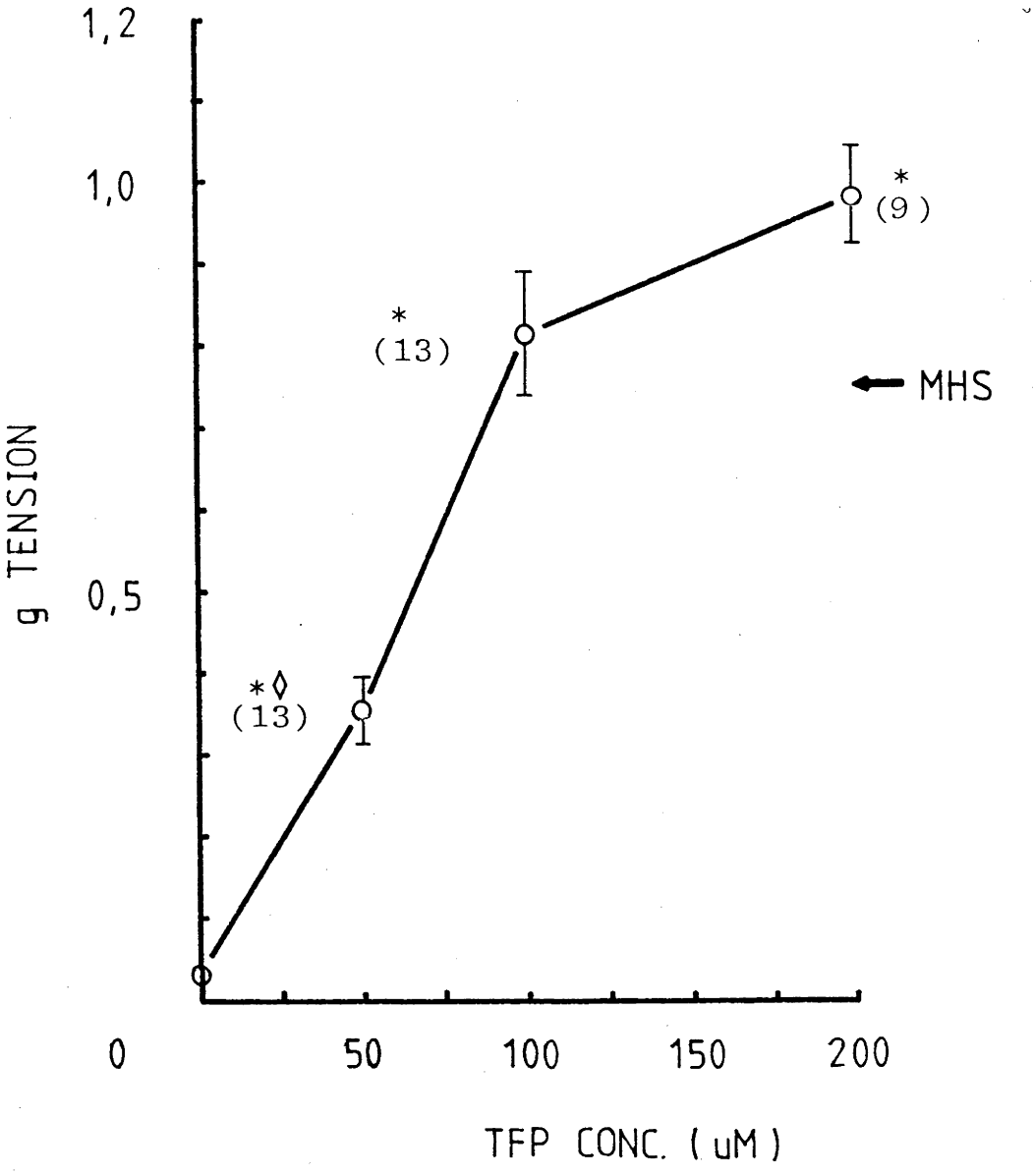


Figure 2.11 Dose Response of Trifluoperazine-Induced Hypercontractility to 3% Halothane in Control Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

* Significantly greater than control muscle response (without trifluoperazine) $p < 0.003$

◊ Significantly less than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.03$.

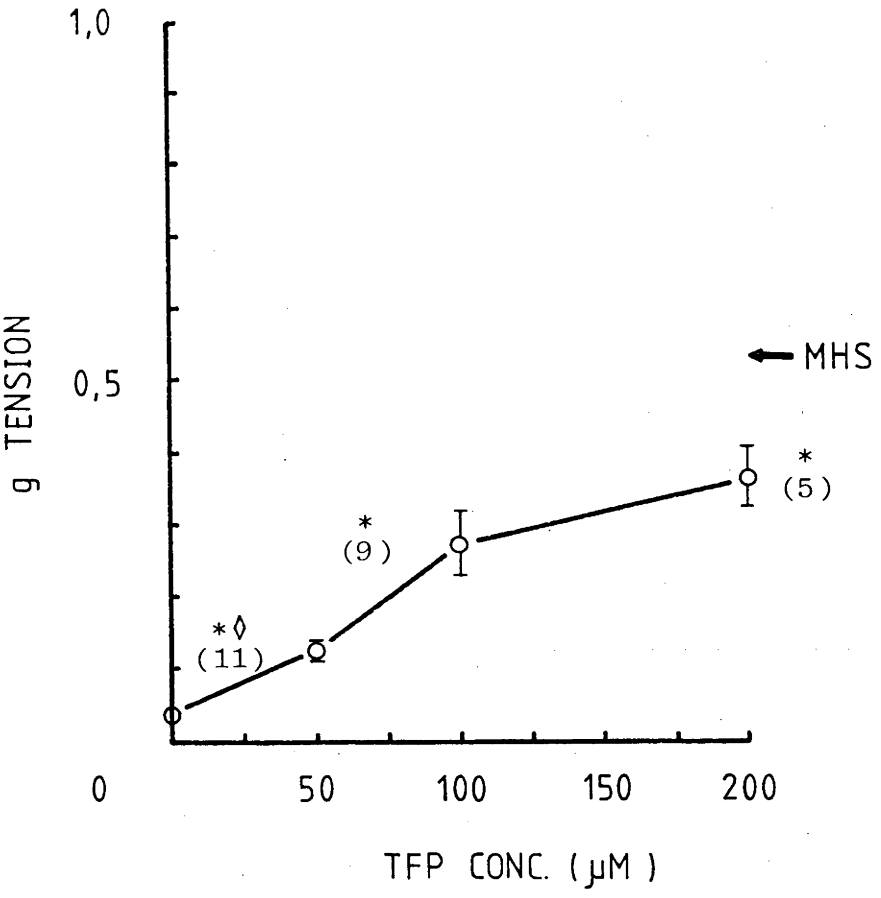


Figure 2.12 Dose Response of Trifluoperazine-Induced Hypercontractility to 2mM Caffeine in Control Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

* Significantly greater than control muscle response (without trifluoperazine) $p < 0.03$

◊ Significantly less than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.005$.

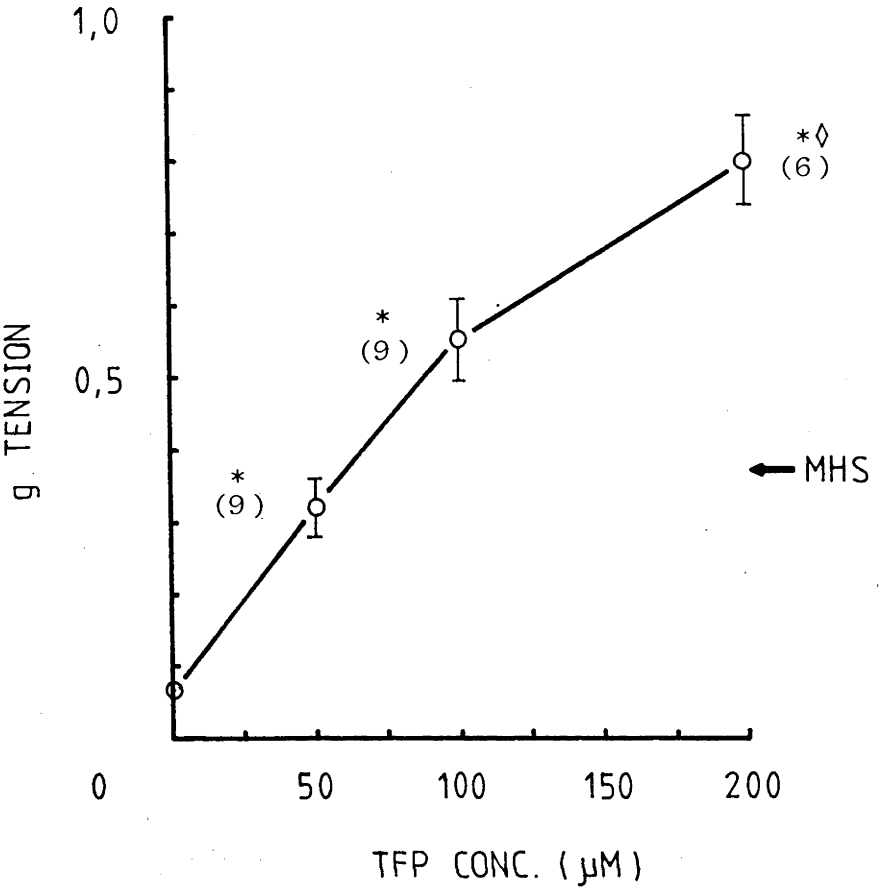


Figure 2.13 Dose Response of Trifluoperazine-Induced Hypercontractility to 1mM Succinylcholine in Control Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

* Significantly greater than control muscle response (without trifluoperazine) $p < 0.002$

◊ Significantly greater than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.003$.

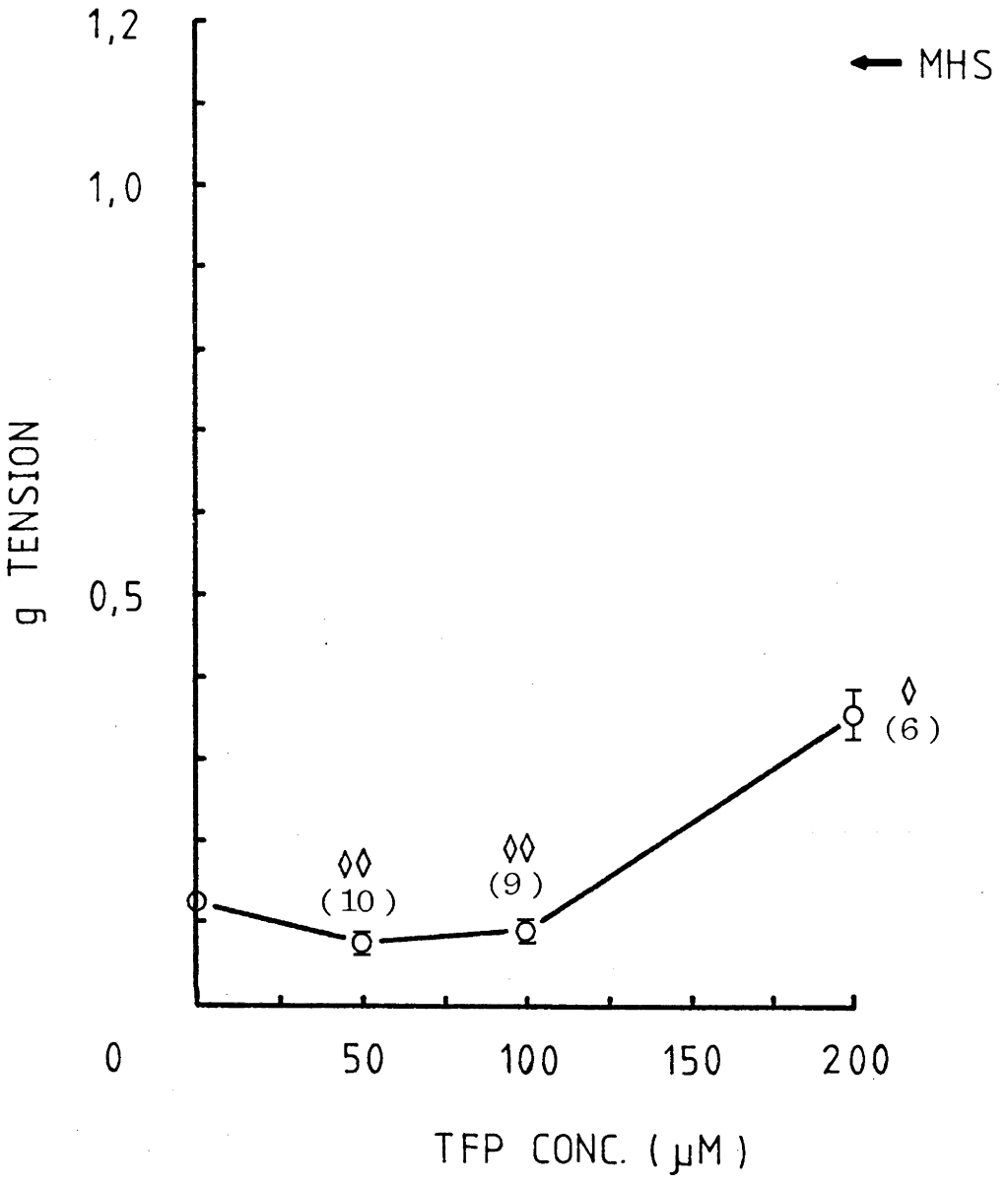


Figure 2.14 Dose Response of Trifluoperazine Effects on 80 mM Potassium Chloride-Induced Contracture in Control Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

◊ Significantly less than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.05$

◊◊ Significantly less than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.001$.

The calmodulin antagonists HPD, CPZ, FPZ and PRO also induced hypercontractility to halothane, caffeine and succinylcholine in control muscle. Sufficient data to permit analysis and comparison with TFP was obtained for HPD and CPZ at a pretreatment concentration of 50 μM (Tables 2.5 and 2.6, respectively). When the abilities of TFP, HPD and CPZ to induce hypercontractility to halothane, caffeine and succinylcholine in control muscle were compared, HPD was the most potent (Figure 2.15). Haloperidol and CPZ induced significantly larger halothane and caffeine contractures when compared with TFP. Also, HPD induced significantly larger succinylcholine contractures in control muscle when compared with TFP (Figure 2.15). Contracture responses to potassium chloride induced by HPD in control muscle were significantly greater than those induced by TFP but were not significantly different from the potassium chloride contracture responses of untreated control muscle (Figure 2.15).

Dantrolene sodium partially reversed (approximately 50%) the calmodulin antagonist induced contracture responses to halothane, caffeine and succinylcholine in control muscle (Figure 2.16).

Table 2.5 Hypercontractility Induced in Control Muscle and Potentiation Induced in Malignant Hyperpyrexia Susceptible Muscle by 50 μM Haloperidol. The number of experiments are shown in brackets.

Mean Tension (g) ± S.E.

	CONTROL	CONTROL + 50 μM HPD	MHS	MHS + 50 μM HPD
HALOTHANE	0.0284 ± 0.006 (93)	***+ 1.6125 ± 0.473 (4)	*** 0.7567 ± 0.045 (187)	***+++ 2.125 ± 0.332 (8)
CAFFEINE	0.0359 ± 0.01 (90)	** 0.7167 ± 0.128 (11)	*** 0.5317 ± 0.036 (149)	*+++ 2.0017 ± 0.456 (6)
SUCCINYLCHOLINE	0.0701 ± 0.021 (79)	***++ 0.864 ± 0.259 (5)	*** 0.3724 ± 0.029 (125)	***+++ 1.7057 ± 0.230 (7)
POTASSIUM CHLORIDE	0.1264 ± 0.06 (80)	0.4986 ± 0.17 (7)	*** 1.1513 ± 0.087 (119)	*** 1.1944 ± 0.218 (9)

* Significantly greater than control muscle response (without haloperidol) $p < 0.009$

** Significantly greater than control muscle response (without haloperidol) $p < 0.004$

*** Significantly greater than control muscle response (without haloperidol) $p < 0.001$

\+ Significantly greater than malignant hyperpyrexia susceptible muscle response (without haloperidol) $p < 0.008$

++ Significantly greater than malignant hyperpyrexia susceptible muscle response (without haloperidol) $p < 0.002$

+++ Significantly greater than malignant hyperpyrexia susceptible muscle response (without haloperidol) $p < 0.001$

Table 2.6 Hypercontractility Induced in Control Muscle and Potentiation Induced in Malignant Hyperpyrexia Susceptible Muscle by 50 μM Chlorpromazine. The number of experiments are shown in brackets.

	Mean Tension (g) ± S.E.			
	CONTROL	CONTROL + 50 μM CPZ	MHS	MHS + 50 μM CPZ
HALOTHANE	0.0284 ± 0.006 (93)	1.226 ± 0.297 (6) **	0.7567 ± 0.045 (187) ***	2.97 ± 0.693 (3) *++
CAFFEINE	0.0359 ± 0.01 (90)	0.41 ± 0.147 (5)	0.5317 ± 0.036 (149) ***	1.4 (2)
SUCCINYLCHOLINE	0.0701 ± 0.021 (79)	0.728 ± 0.236 (10) ***+	0.3724 ± 0.029 (125) ***	1.856 ± 0.507 (5) ***++
POTASSIUM CHLORIDE	0.1264 ± 0.06 (80)	0.191 ± 0.101 (7) #	1.1513 ± 0.087 (119) ***	0.605 (2)

* Significantly greater than control muscle response (without chlorpromazine) $p < 0.05$

** Significantly greater than control muscle response (without chlorpromazine) $p < 0.005$

*** Significantly greater than control muscle response (without chlorpromazine) $p < 0.001$

\+ Significantly greater than malignant hyperpyrexia susceptible muscle response (without chlorpromazine) $p < 0.005$

++ Significantly greater than malignant hyperpyrexia susceptible muscle response (without chlorpromazine) $p < 0.001$

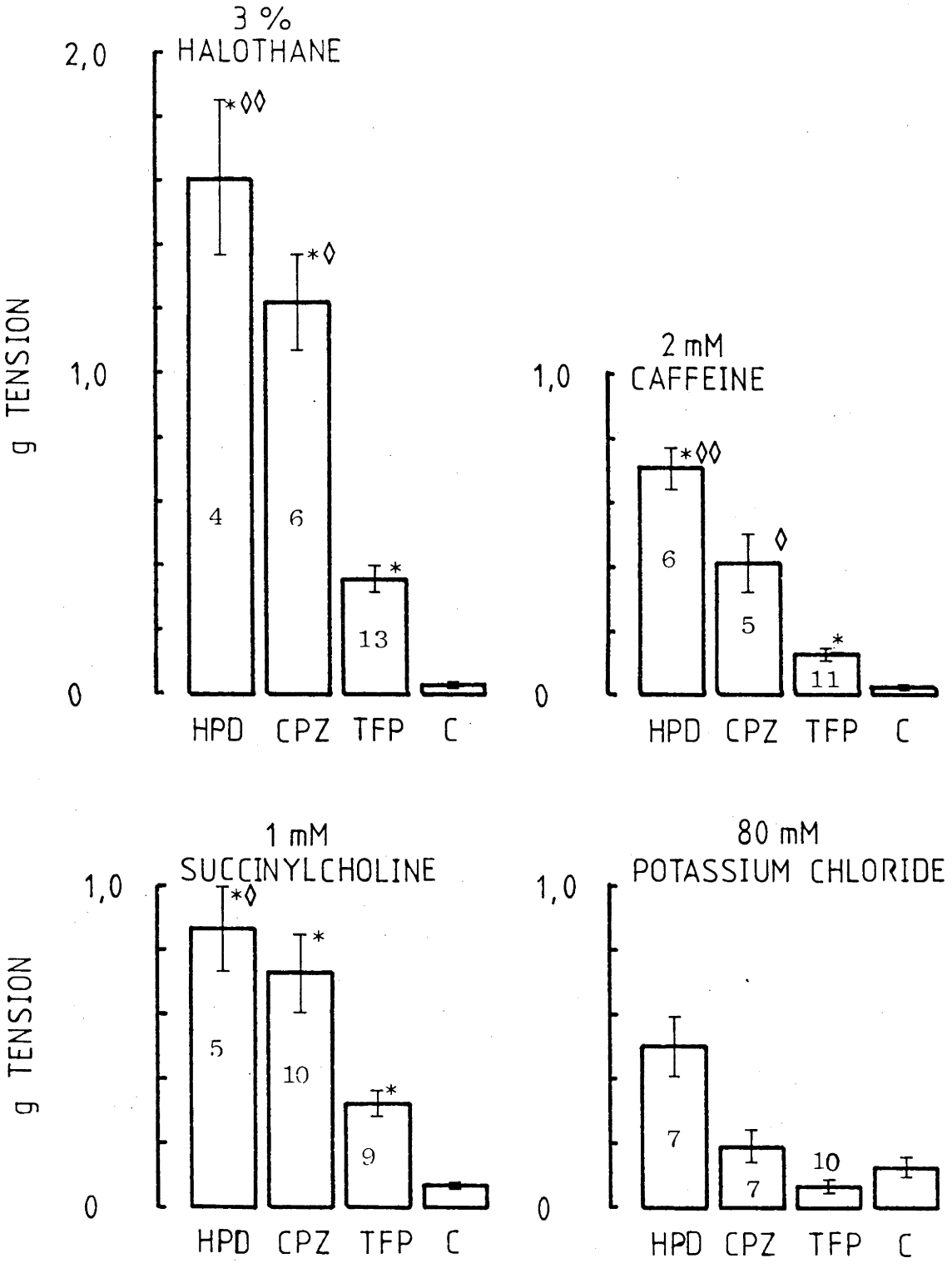
\# Significantly less than malignant hyperpyrexia susceptible muscle response (without chlorpromazine) $p < 0.01$

Figure 2.15 Comparison of 50 μM Haloperidol, 50 μM Chlorpromazine and 50 μM Trifluoperazine-Induced Hypercontractility to Halothane, Caffeine and Succinylcholine in Control Muscle. The results shown are mean values ± S.E.

* Significantly greater than control muscle response (without trifluoperazine) $p < 0.005$

◊ Significantly greater than control + 50 μM trifluoperazine response $p < 0.05$

◊◊ Significantly greater than control + 50 μM trifluoperazine response $p < 0.002$


3 %
HALOTHANE
2,0
1,0
0
g TENSION
*◊◊
*◊
*
4
6
13
HPD
CPZ
TFP
C
2 mM
CAFFEINE
1,0
0
*◊◊
◊
*
6
5
11
HPD
CPZ
TFP
C
1 mM
SUCCINYLCHOLINE
1,0
0
g TENSION
*◊
*
*
5
10
9
HPD
CPZ
TFP
C
80 mM
POTASSIUM CHLORIDE
1,0
0
7
7
10
HPD
CPZ
TFP
C

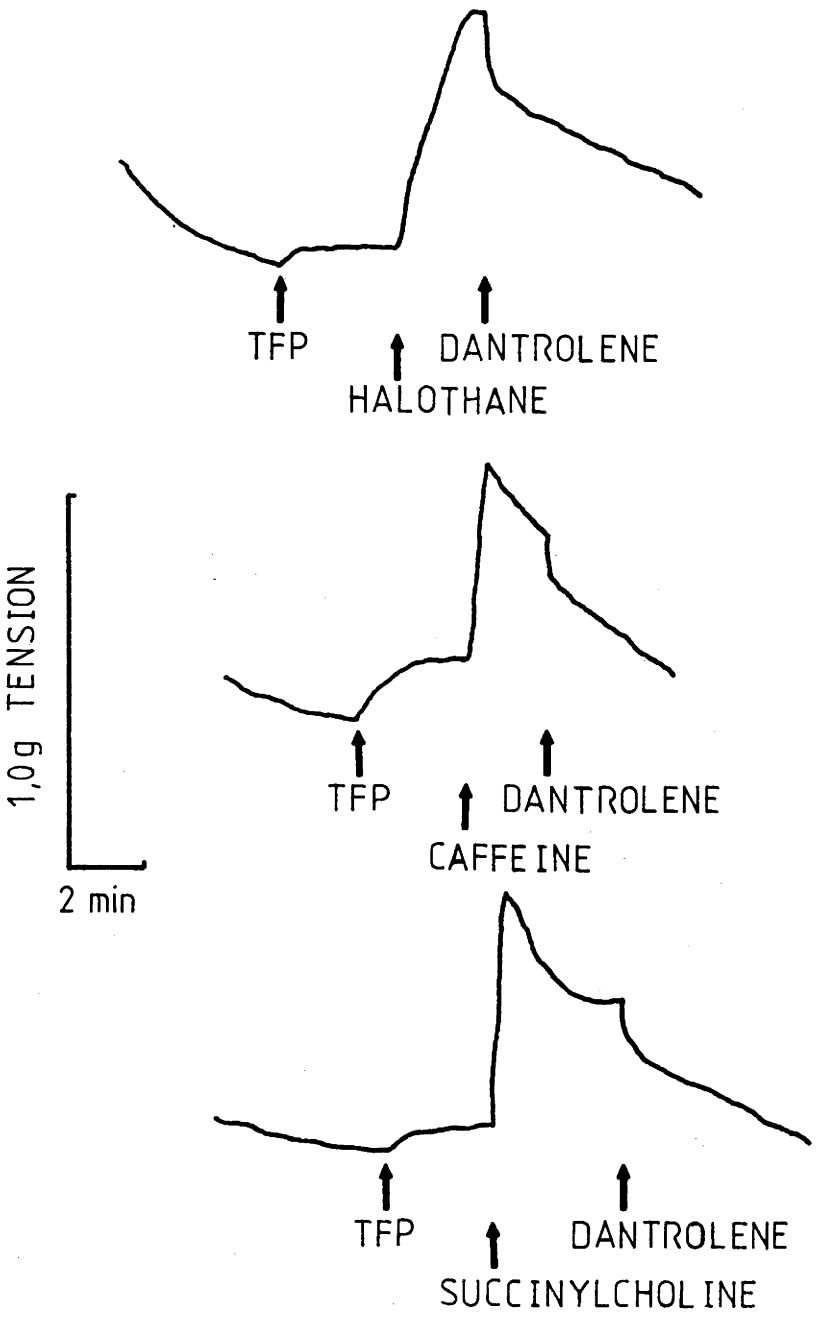


Figure 2.16 Reversal of 100 μM Trifluoperazine-Induced 3% Halothane, 2 mM Caffeine and 1 mM Succinylcholine Contractures by 6 μM Dantrolene Sodium in Control Muscle.

2.3.5 The Effects of Calmodulin Antagonist Drugs on the Contractures Induced by Halothane, Caffeine, Succinylcholine and Potassium Chloride in Malignant Hyperpyrexia Susceptible Porcine Muscle.

The calmodulin antagonists TFP, HPD and CPZ potentiated contractures induced by halothane, caffeine and succinylcholine in MHS muscle. The calmodulin antagonist drugs had no effect on potassium chloride induced contractures in MHS muscle. When pretreated with 50 μM TFP, MHS muscle responded to 3% halothane with a mean contracture approximately twice as large as the mean halothane contracture in untreated MHS muscle (Table 2.4). Greater concentrations of TFP (100 μM and 200 μM) did not significantly increase this amount of contracture (Figure 2.17). The mean contracture responses of MHS muscle pretreated with 50 μM TFP induced by 2mM caffeine and 1mM succinylcholine were approximately doubled when compared to the mean contracture of untreated MHS muscle (Table 2.4 and Figure 2.18). Haloperidol and CPZ also potentiated MHS muscle contracture responses to halothane, caffeine and succinylcholine (Tables 2.5 and 2.6 and Figure 2.18). The potentiating effects of HPD were significantly greater than those of TFP in MHS muscle.

Dantrolene sodium reversed the calmodulin antagonist-potentiated contracture responses to halothane, caffeine and succinylcholine in MHS muscle (Figure 2.19).

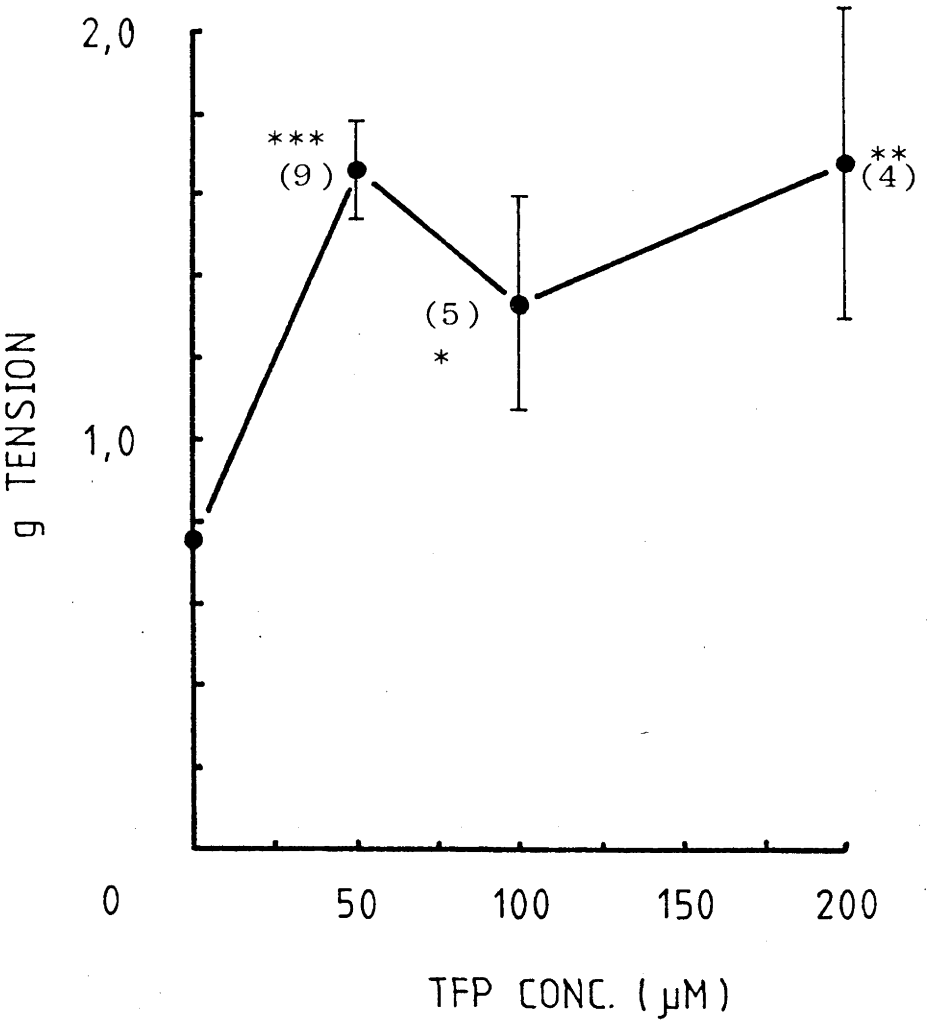


Figure 2.17 Dose Response of Trifluoperazine-Induced Potentiation of Halothane Contracture in Malignant Hyperpyrexia Susceptible Muscle. The results shown are mean values ± S.E. The number of experiments appears in brackets.

* Significantly greater than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.05$

** Significantly greater than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.005$

*** Significantly greater than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.001$

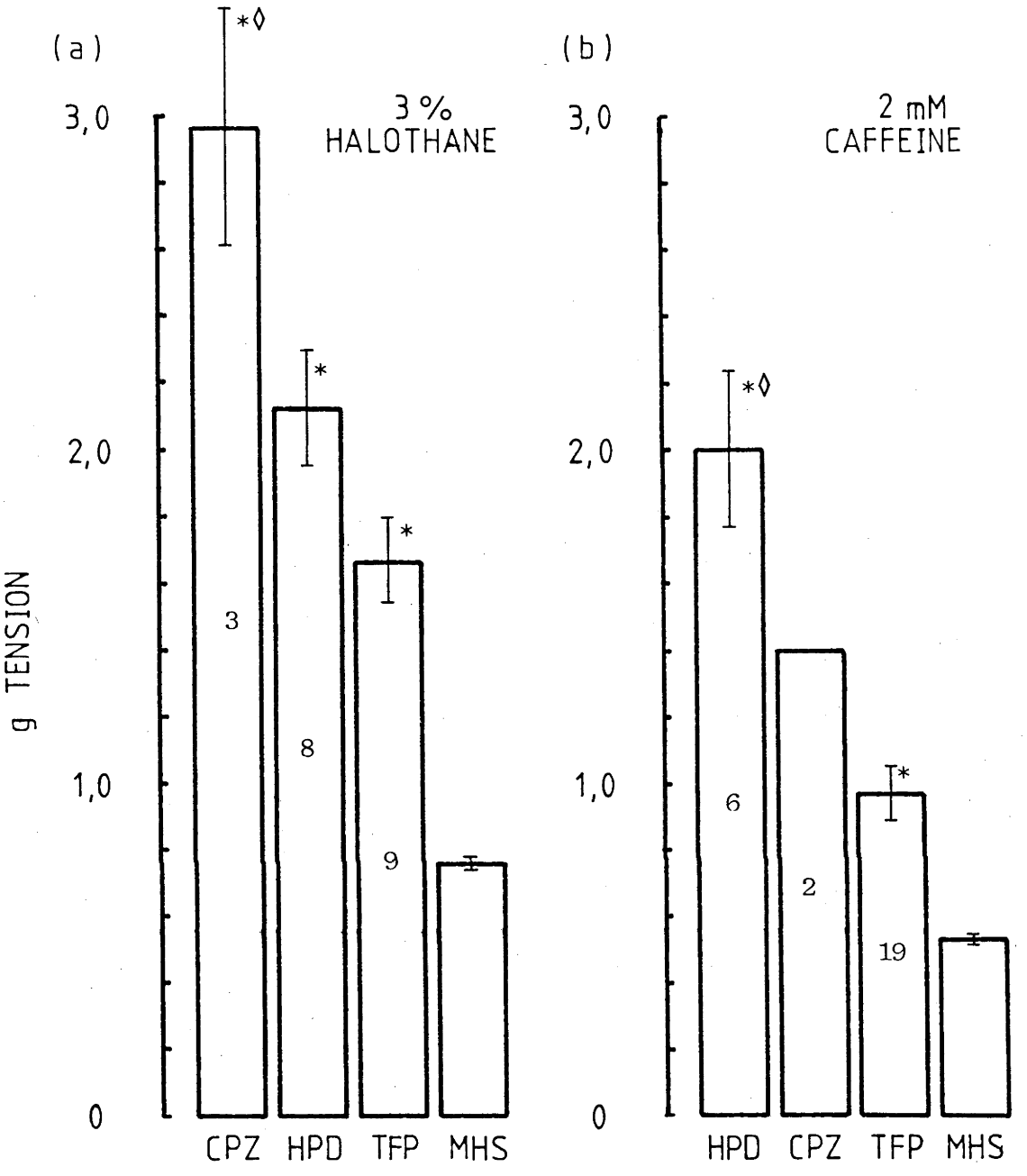
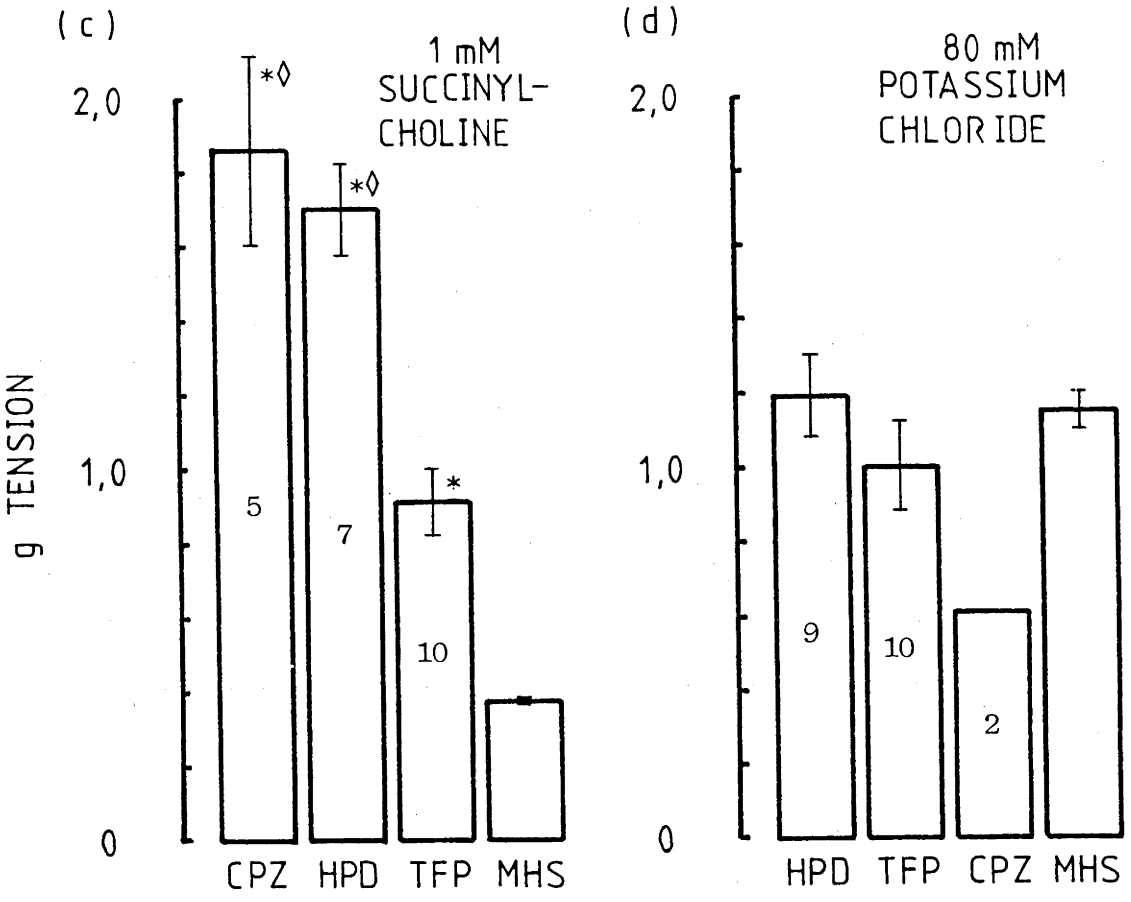


Figure 2.18 Comparison of 50 μM Haloperidol, 50 μM Chlorpromazine and 50 μM Trifluoperazine-Induced Potentiation of Halothane, Caffeine and Succinylcholine Contractures in Malignant Hyperpyrexia Susceptible Muscle. The results shown are mean values ± S.E.

(Figure 2.18 continued overleaf)



* Significantly greater than malignant hyperpyrexia susceptible muscle response (without trifluoperazine) $p < 0.001$

◊ Significantly greater than malignant hyperpyrexia susceptible muscle + 50 μM trifluoperazine $p < 0.005$

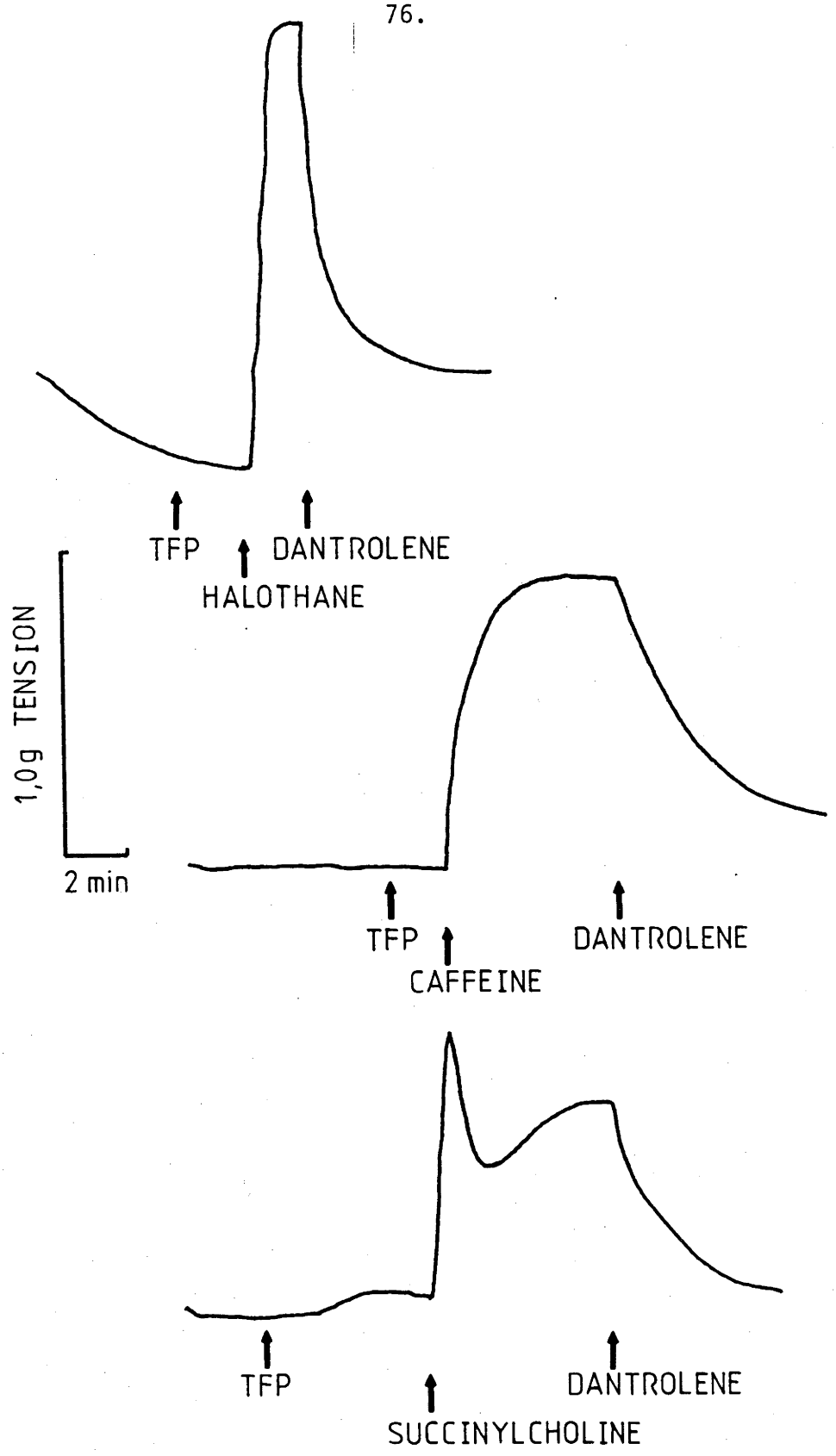


Figure 2.19 Reversal of 50 μM Trifluoperazine-Potentiated 3% Halothane, 2 mM Caffeine and 1 mM Succinylcholine Contractures by 6 μM Dantrolene Sodium in Malignant Hyperpyrexia Susceptible Muscle.

2.4 Discussion

The results obtained in this chapter suggested that the calmodulin antagonist drugs were affecting some aspect of muscle cell Ca^{2+} metabolism which led to a rise in myoplasmic Ca^{2+} concentrations in both control and MHS muscle. The ability of these drugs to increase the myoplasmic Ca^{2+} concentration of control muscle induced a MH-like hypercontractility to halothane, caffeine and succinylcholine in this tissue. This observation was consistent with the view that MHS muscle hypercontractility was caused by an abnormally high myoplasmic Ca^{2+} concentration. Also, since the calmodulin antagonist drugs were able to affect the contractile characteristics of both control and MHS muscle, calmodulin did not appear to play a unique role in MHS muscle hypercontractility.

The hypothesis that the calmodulin antagonist drugs induced alterations in the contractile characteristics of skeletal muscle *in vitro* by inducing an increase in the myoplasmic Ca^{2+} concentration was consistent with a number of observations. The drugs TFP, HPD, CPZ, FPZ and PRO induced *in vitro* contractures in control and MHS muscle. The drug which was studied in the greatest detail was TFP. The contractures induced by TFP in MHS muscle at concentrations of 50 µM, 100 µM, 200 µM and 500 µM were significantly greater than those induced in control muscle at these concentrations. This difference might be explained by the abnormally high myoplasmic Ca^{2+} concentration thought to be present in resting MHS muscle. Trifluoperazine, HPD and CPZ also induced hypercontractility to halothane, caffeine and succinylcholine in control muscle and potentiated the hypercontractility of MHS muscle to these agents.

The inability of the calmodulin antagonists to induce potassium chloride contracture in control muscle may be due to an electrical stabilization of the sarcolemma by these drugs. Chlorpromazine and other hydrophobic drugs have been shown to reduce the excitability of excitable membranes (Langslet, 1970; Seeman, 1972). Electrical stabilization of the control muscle sarcolemma may prevent depolarization by 80 mM potassium chloride and therefore prevent potassium chloride contracture. The inability of the calmodulin antagonist drugs to potentiate potassium chloride contractures in MHS muscle appears to be more complex. Let us assume that the MHS sarcolemma was stabilized to the same degree as the control sarcolemma by calmodulin antagonist drugs, and that this stabilization was not absolute. In control muscle, the residual depolarization caused by potassium chloride in the presence of calmodulin antagonist drugs was insufficient to induce contracture. In MHS muscle, where the abnormally high myoplasmic Ca^{2+} concentration had been further increased by the action of the calmodulin antagonist drug, the residual depolarization might induce a contracture which was not significantly different to an untreated MHS muscle potassium chloride contracture. Alternatively, the ability of the MHS sarcolemma to be depolarized in the presence of electrical stabilization by calmodulin antagonist drugs may be due to an abnormality of the MHS sarcolemma such as that described by Gallant, Godt and Gronert (1979). These authors observed that halothane depolarized the sarcolemma of MHS muscle but not control muscle. The observations made in the present study may suggest a basic difference in the mode of action of potassium chloride in skeletal muscle when compared to halothane, caffeine and succinylcholine.

The most potent calmodulin antagonist drugs tested in the in vitro muscle strip experiments (PEN and PIM) failed to induce the effects observed with other antagonist drugs. The failure of PEN and PIM to induce effects in skeletal muscle strips may be due to their insolubility in the aqueous organ bath environment (octanol/water partition coefficients 40,000,000 and 2,000,000, respectively).

The mechanism by which calmodulin antagonist drugs affect myoplasmic Ca^{2+} metabolism may include a number of sites of action. Of the known calmodulin-dependent functions in skeletal muscle (Chapter 1.11.3), two may have contributed to this effect. Firstly, the calmodulin-dependent phosphorylation of one of the three myosin light chains by myosin light chain kinase (MLCK) (Mayr and Heilmeyer, 1983) may be involved. Although no definition of MLCK function has been described, cycles of phosphorylation and dephosphorylation of this myosin light chain have been observed in response to contraction and relaxation (Manning and Stull, 1979). It has been proposed by Crow and Kushnerick (1982) that light chain phosphorylation may regulate the rate of cross-bridge turnover. If this proposal was correct, however, inhibition of calmodulin function by calmodulin antagonist drugs would be expected to favour muscle relaxation, not contraction. Secondly, a calmodulin-dependent protein phosphorylation system of the SR membrane has been proposed to be involved in the regulation of the release of Ca^{2+} from the SR (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982, 1983). Although the function of these protein phosphorylations has not been elucidated, inhibition of this calmodulin dependent function by calmodulin antagonist drugs might lead to a disruption of the regulation of Ca^{2+} release from the SR resulting in a rise in the myoplasmic Ca^{2+} concentration.

The published inhibitory potencies and octanol/water partition coefficients of the calmodulin antagonist drugs used in the present study (Table 2.1) did not always correlate with their pharmacological potencies in vitro (Figure 2.9). Promethazine was the least potent calmodulin antagonist and the least hydrophobic drug tested, and it did not induce contracture in control or MHS muscle until high concentrations were reached. Trifluoperazine, however, was approximately six times more potent an inhibitor of calmodulin function than HPD, yet HPD was at least as potent, if not more potent, in producing contracture in control and MHS muscle (Figure 2.9), in inducing hypercontractility in control muscle (Figure 2.15) and in potentiating hypercontractility in MHS muscle (Figure 2.18). These observations suggest that a mode of action other than calmodulin inhibition may be important in the effects of calmodulin antagonist drugs on skeletal muscle in vitro.

Calmodulin antagonist drugs have been shown to bind to troponin C (Levin and Weiss, 1978). At concentrations greater than 10 μM TFP, the extent of TFP binding to calmodulin and troponin C was similar. Since troponin C confers Ca^{2+} sensitivity on the contractile elements in muscle, an increase in the sensitivity of troponin C to Ca^{2+}, or an activation of troponin C in the absence of Ca^{2+}, brought about by the binding of a calmodulin antagonist drug might produce effects similar to those of an increased myoplasmic Ca^{2+} concentration. Takagi (1981) found that in skinned muscle fibres of the guinea pig the addition of CPZ made the contractile system more sensitive to Ca^{2+}. Such an effect of calmodulin antagonist drugs may have contributed to their effects in control and MHS muscle in the present study.

Due to the high lipid solubility of the calmodulin antagonist drugs, effects on the sarcolemma and the SR would seem likely at the concentrations used in the organ bath. Chlorpromazine has been found to inhibit the active Ca^{2+} uptake by isolated SR (Balzer, Makinose and Hasselbach, 1968) and the influx and efflux of Ca^{2+} through the sarcolemma (Balzer and Hellenbrecht, 1969). Andersson (1972) and Takagi (1981) both concluded that the major contractile effects produced by CPZ in skeletal muscle fibres were mediated by its action upon the SR, releasing Ca^{2+}.

Contractures produced by TFP and HPD in control and MHS muscle differed in the degree to which they were reversed by dantrolene sodium (Table 2.3). Haloperidol contractures were reversed by dantrolene sodium by approximately 78% whereas TFP contractures were reversed by dantrolene sodium by approximately 20%. This difference might be due to HPD being more active than TFP at a dantrolene sodium sensitive site. It might also be explained if these two drugs both affected the same dantrolene sodium-sensitive site but TFP, being more hydrophobic than HPD, was harder for dantrolene sodium to displace (Table 2.1). The dantrolene sodium-sensitive site in skeletal muscle is thought to be excitation-contraction coupling (E-C coupling) (Ellis and Bryant, 1972). Dantrolene sodium does not stimulate the Ca^{2+}-dependent ATPase of the SR or affect Ca^{2+} exchange or efflux rates of the SR (White, Collins and Denborough, 1983).

2.5 Summary

The effects of calmodulin antagonist drugs on control and MHS muscle *in vitro* did not reveal any unique or specific role for calmodulin in the hypercontractility of MHS muscle. The calmodulin antagonist

drugs induced contracture in both control and MHS muscle strips, induced hypercontractility to halothane, caffeine and succinylcholine in control muscle, and potentiated hypercontractility to halothane, caffeine and succinylcholine in MHS muscle. These effects were probably due to a rise in the concentration of myoplasmic Ca^{2+} caused by the calmodulin antagonist drugs in both control and MHS muscle. This increase in myoplasmic Ca^{2+} concentration may have been due to the calmodulin antagonist drugs releasing Ca^{2+} from the SR by acting on the calmodulin-dependent protein kinase system of the SR or by calmodulin-independent, non-specific interactions with the SR membrane. Other mechanisms by which calmodulin antagonist drugs might effect the contractile characteristics of control and MHS muscle include binding to troponin C thereby causing an increase in the sensitivity of the contractile elements to Ca^{2+}, and by acting on the E-C coupling mechanism.

CHAPTER 3 CALMODULIN IN PORCINE MALIGNANT HYPERPYREXIA

3.1 Introduction

The intracellular calcium binding protein calmodulin plays a major role in mediating the effects of changes in Ca^{2+} levels in many types of cells (Chapter 1.4). The Ca^{2+}-calmodulin complex is able to bind to its effector proteins and bring about a change in their activities. One of the major roles played by calmodulin in some cells (for example, the erythrocyte) is to regulate the intracellular levels of Ca^{2+}. That is, it is able to regulate Ca^{2+} transport across some membranes. Since calmodulin plays these multiple roles in cellular Ca^{2+} regulation and mediation, and since the behaviour of skeletal muscle from MHS individuals and swine *in vitro* suggests an abnormality in some aspect of Ca^{2+} regulation (Denborough, 1980), it was decided to investigate the role of calmodulin in MH.

The first step in this investigation involved the pharmacological effects of calmodulin antagonist drugs on porcine skeletal muscle strips *in vitro*. This work is described in the previous chapter. Briefly, these drugs caused control swine skeletal muscle to behave in a similar manner to muscle from MHS swine, that is, they induced a hypercontractility to halothane, caffeine and succinylcholine. Dantrolene sodium partially reversed the contractures which were produced. The hypercontractility of MHS muscle was potentiated by the calmodulin antagonist drugs.

Because of the effects induced by calmodulin antagonists in skeletal muscle strips, the pharmacological work was extended to the isolation

of calmodulin from control and MHS porcine brain. These preparations were then compared both functionally and physically to determine if they differed in any way.

3.2 Materials and Methods

3.2.1 Drugs and Reagents

The following gifts of drugs are gratefully acknowledged: TFP from Smith, Kline and French Laboratories (Australia), FPZ from E.R. Squibb and Sons, and HPD from Searle Laboratories.

All other reagents were of analytical grade.

3.2.2 Isolation of Calmodulin from Control and Malignant Hyperpyrexia Susceptible Porcine Brain

Calmodulin preparations were isolated from both control and MHS porcine brains essentially by the method of Kakiuchi, Sobue, Yamazaki, Kambayashi, Sakon and Kosaki (1981). The brains were removed immediately post-mortem, placed into liquid nitrogen and stored at -70^{o}C until used.

All procedures were carried out between 0^{o} and 4^{o}C. Frozen brain tissue (70 - 110 g) was homogenized in approximately 4 volumes of 4% trichloro-acetic acid (TCA) and then stirred slowly in the cold for 20 minutes. This mixture was centrifuged at 20,000xg for 30 minutes and the pellet homogenized in 250 ml of 20 mM Tris (hydroxymethyl) aminomethane (Tris; pH 7.5), 5 mM ethylenediaminotetra-acetic acid (EDTA), 0.1 mM phenylmethylsulfonylfluoride (PMSF), 0.05 µg/ml pepstatin A and 2 µg/ml antipain (Buffer A). After adjusting the pH

of this homogenate to 7.5, it was centrifuged at 100,000xg for 2 hours. The pellets were washed with the same buffer and the supernatants were combined. These supernatants were then applied to a diethylaminoethyl-cellulose (DEAE-cellulose) column (2.5 x 32 cm) equilibrated with 20 mM Tris (pH 7.5), 5 mM 2-mercaptoethanol and 0.1 mM ethyleneglycoltetra-acetic acid (EGTA). The column was eluted with this buffer plus 0.145 M and 0.3 M $(NH_4)_2SO_4$ in a stepwise manner. Fractions of 8 ml were collected. Those fractions containing calmodulin activity were combined and diluted with an equal volume of 20 mM Tris (pH 7.5), 5 mM 2-mercaptoethanol and 0.5 mM $CaCl_2$ and applied to an affinity column of FPZ coupled to Sepharose 6B which had been equilibrated with 20 mM Tris (pH 7.5), 5 mM 2-mercaptoethanol, 500 mM NaCl and 0.2 mM $CaCl_2$. The column was eluted with this buffer, then with the same buffer except that the $CaCl_2$ was replaced by 2 mM EGTA. Fractions of 4 ml were collected, and those containing calmodulin activity were combined, dialysed against 10 mM Tris (pH 7.0), and lyophilized.

3.2.3 The Assay of Calmodulin Activity

Calmodulin activity was measured by the method of Teo, Wang and Wang (1973) with minor modifications. The sample for assay was mixed with 0.016 units of calmodulin-deficient cyclic-AMP phosphodiesterase (Sigma Chemical Company, product number P-0520) and 0.2 units of 5' nucleotidase (Sigma Chemical Company, product number N-4005) in 0.5 ml and equilibrated at 37°C. The reaction was initiated by the addition of 0.5 ml of 72 mM Tris (pH 7.5), 72 mM imidazole, 36 mM Mg acetate, 1.8 mM cyclic-AMP and 100 µM $CaCl_2$. The reaction was

terminated after 30 minutes by the addition of 4 ml of inorganic phosphate reagent.

The calmodulin inhibition studies were carried out as above with a standard amount of calmodulin (100 ng).

3.2.4 The Assay of Porcine Skeletal Muscle Cyclic Adenosine 3',5'-monophosphate

Samples of gracilis muscle were obtained by biopsy under pentothal/N_2O anaesthesia, immediately frozen in liquid nitrogen and stored at -70^{o}C until used. Approximately 0.5 g (wet weight) of muscle tissue was homogenized in 7% TCA using a Polytron homogenizer. An aliquot of this homogenate was taken for protein determination. The remainder was centrifuged at 3,000xg for 15 minutes and then the supernatant was extracted 3 times with 2-3 volumes of water-saturated ether to remove the TCA. The extracted supernatant was then bubbled with nitrogen for 5-10 minutes. An aliquot was then taken for analysis by a Radiochemical Centre, Amersham (Buckinghamshire, England) Cyclic AMP assay kit (code TRK. 432).

3.2.5 Ultraviolet Absorption Spectrum of Calmodulin

Ultraviolet absorption spectra of calmodulin preparations were performed using a Beckman DU-8 spectrophotometer and wavelength scan module. The wavelengths examined were 240 - 320 nm, and the protein concentration was 0.5 mg/ml in 0.1 M imidazole and 1 mM EDTA (pH 7.0).

3.2.6 Polyacrylamide Gel Electrophoresis of Proteins

Polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (1970), using 10 - 20% acrylamide gradient slab gels with a 4.5% acrylamide stacking gel. Gels were run at 40 ma/gel and stained with Coomassie blue.

3.2.7 Inorganic Phosphate Determinations

Inorganic phosphate was determined by the method of Heinonen and Lahti (1981).

3.2.8 Protein Determinations

Protein concentrations were determined by a modification of the Lowry method (Peterson, 1977).

3.3 Results

3.3.1 Isolation of Calmodulin from Control and Malignant Hyperpyrexia Susceptible Porcine Brain

In order to compare the physical and functional characteristics of calmodulin from control and MHS swine, preparations of this protein were extracted from porcine brains. Although we were interested in calmodulin function in skeletal muscle, brain preparations were used since calmodulin lacks tissue specificity (Kakiuchi, Sobue, Yamazaki, Kambayashi, Sakon and Kosaki, 1981) and brain is the richest source of calmodulin (Smoake, Song and Cheung, 1974). The isolation procedure utilized a TCA extraction of whole brain followed by DEAE-cellulose chromatography. A subsequent affinity chromatography step utilized one of the phenothiazines known to bind to calmodulin in a Ca^{2+}-dependent manner (fluphenazine) coupled to Sepharose 6B

(Figures 3.1, 3.2 and 3.3). The calmodulin containing eluate from the DEAE-cellulose column was applied to the phenothiazine affinity column in the presence of Ca^{2+} under conditions where calmodulin would bind to the column. Elution of the bound calmodulin was carried out in the presence of EGTA. Extraction of control and MHS brains gave similar elution profiles and protein compositions.

3.3.2 Sodium Dodecyl Sulphate Electrophoretic Comparison of Calmodulins Isolated from Control and Malignant Hyperpyrexia Susceptible Porcine Brain

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of calmodulins isolated from control and MHS porcine brain showed no difference between these two proteins (Figure 3.4). The calmodulin band, running at approximately 17,000 MW, is accompanied by a number of contaminants of both higher and lower MW. Commercially available porcine brain calmodulin (Sigma Chemical Company), isolated using a method similar to the present study, also contains contaminants in the 19,000 to 21,000 MW range. The low MW contaminant (approximately 14,000 MW) are not present in the commercial preparation. They may be breakdown products of calmodulin accumulated after extended storage.

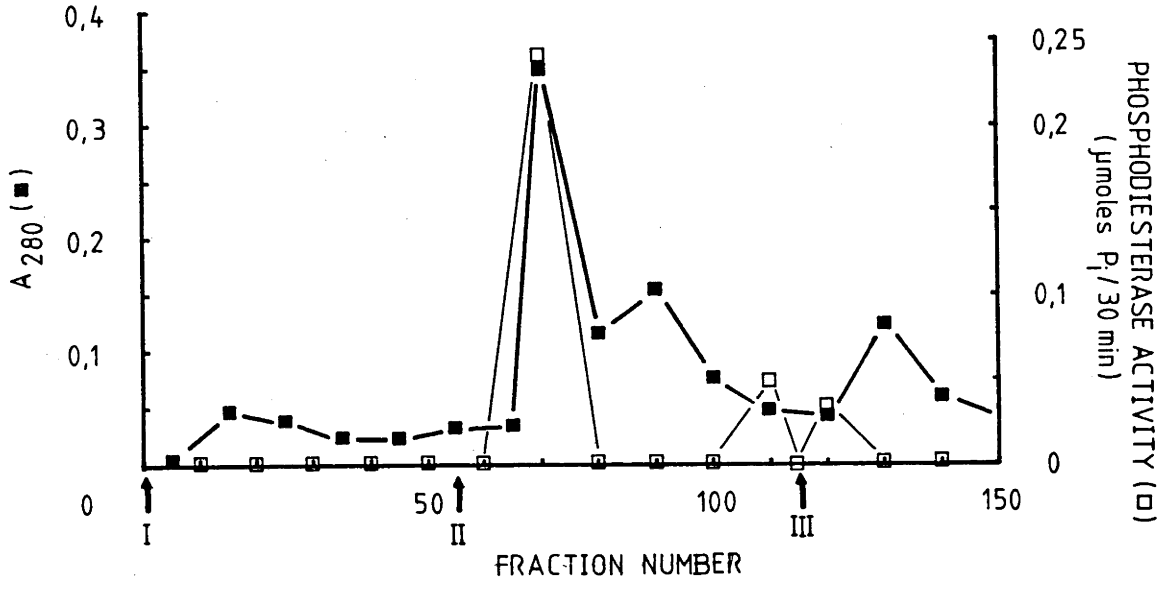


Figure 3.1 The Elution Profile of Protein and Calmodulin Activity from the DEAE-Cellulose Column used in the Isolation of Calmodulin from Malignant Hyperpyrexia Susceptible Porcine Brain.

I. Sample applied.

II. Elution buffer plus 0.145 M $(NH_4)_2\ SO_4$.

III. Elution buffer plus 0.3 M $(NH_4)_2\ SO_4$.

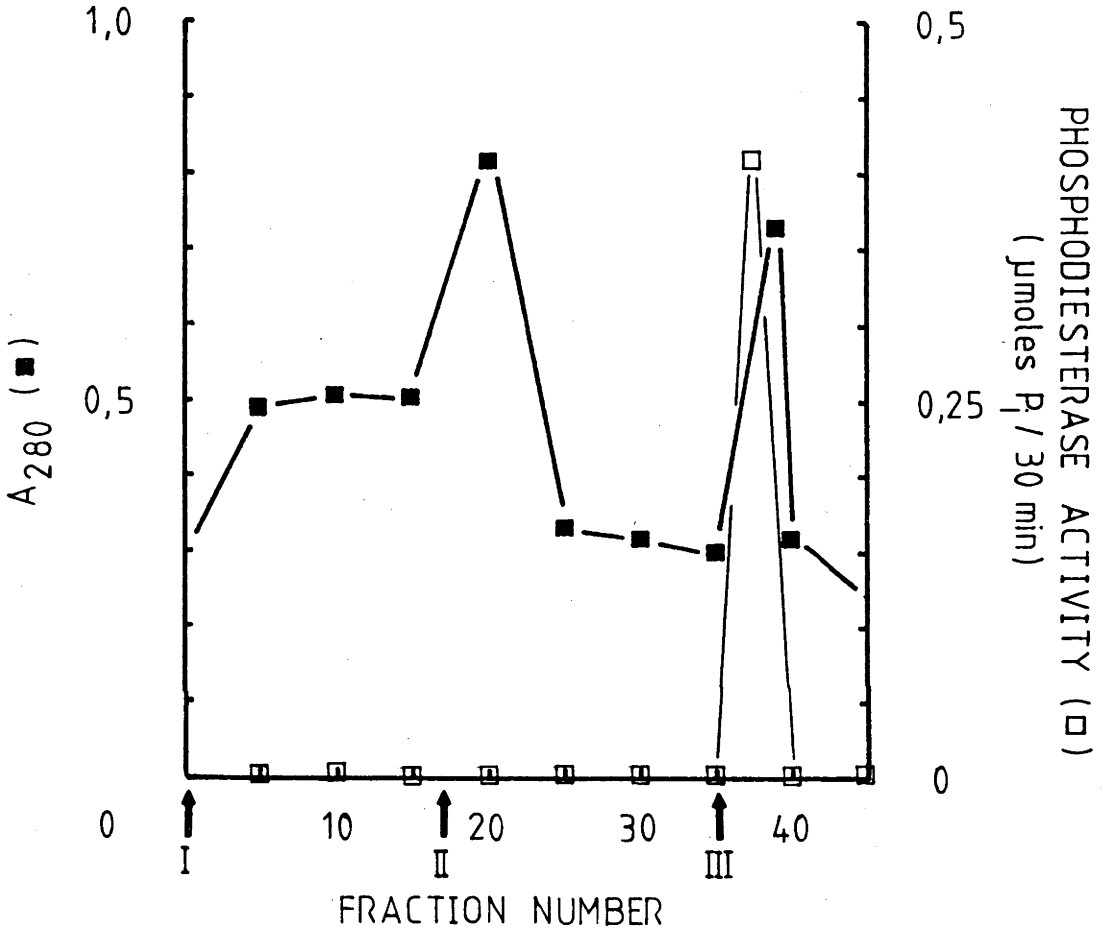


Figure 3.2 Elution Profile of Protein and Calmodulin Activity from the Affinity Column used in the Isolation of Calmodulin from Malignant Hyperpyrexia Susceptible Porcine Brain.

I. Sample applied.

II. Elution buffer.

III. Elution buffer plus 2 mM EGTA

Figure 3.3 Polyacrylamide Gel Pattern of Various Fractions obtained during the Isolation of Calmodulin from Malignant Hyperpyrexia Susceptible Porcine Brain.

A. TCA homogenate

B. Buffer A extract

C. 0.3M $(NH_4)_2SO_4$ DEAE-cellulose column eluate

D. 2mM EGTA affinity column eluate.

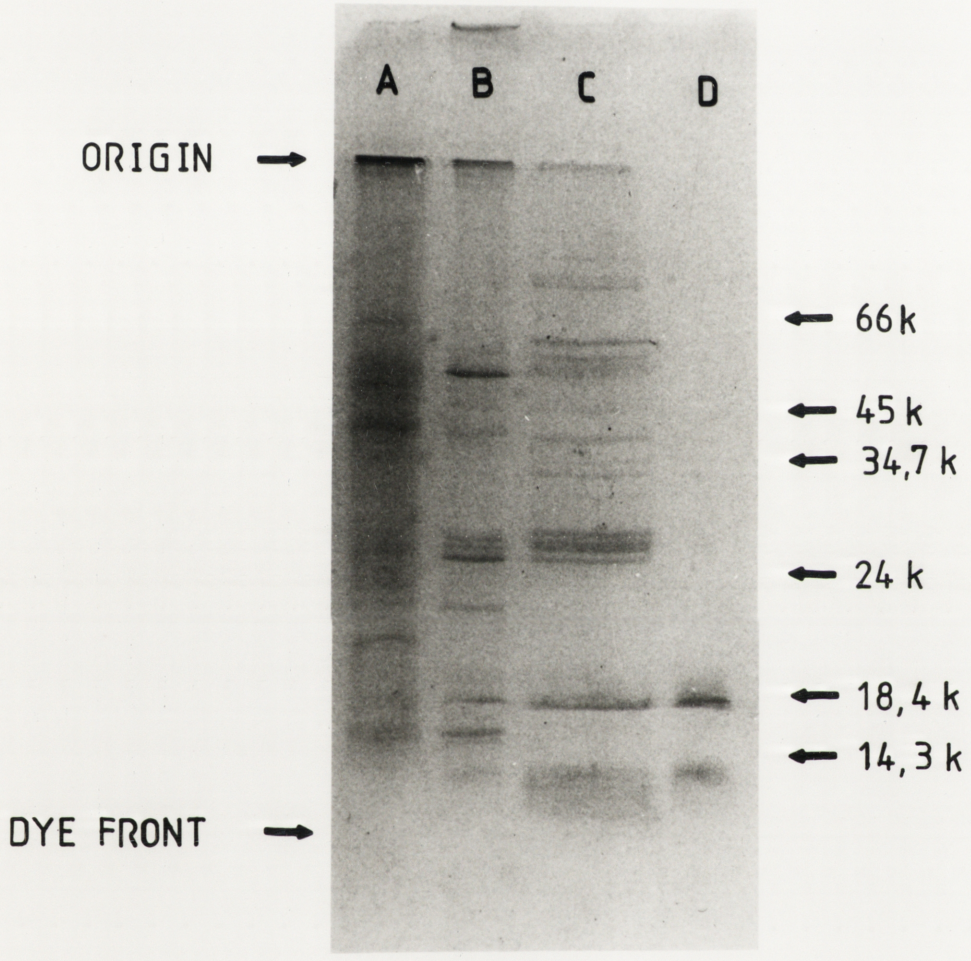
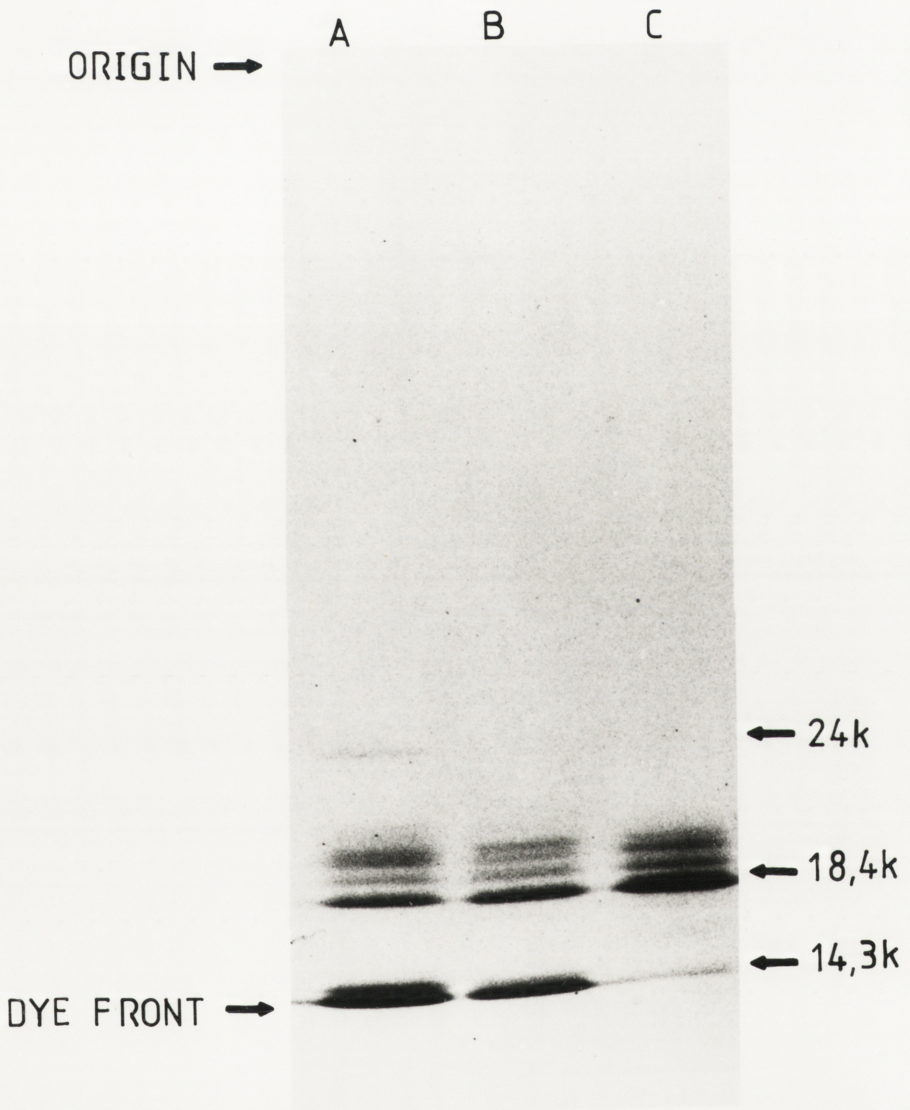

A
B
C
D
ORIGIN
66 k
45 k
34,7 k
24 k
18,4 k
14,3 k
DYE FRONT

Figure 3.4 Comparison of Polyacrylamide Gel Patterns of Calmodulin Isolated from Control and Malignant Hyperpyrexia Susceptible Porcine Brain.

A. Control

B. MHS

C. Commercially available porcine brain calmodulin (Sigma Chemical Company)


A
B
C
ORIGIN
24k
18,4k
14,3k
DYE FRONT

3.3.3 Comparison of the Ultraviolet Absorption Spectra of Calmodulin Isolated from Control and Malignant Hyperpyrexia Susceptible Porcine Brain

Calmodulin exhibits a UV absorption spectrum similar to that of troponin C with a major peak at approximately 280 nm and a trough at approximately 250 nm (Figure 3.5). The multiple shoulders between 250 and 280 nm are characteristic of the fine structure of the absorption band of phenylalanine (Klee, 1977; Waterson, Harrelson, Keller, Sharief and Vanaman, 1976). The UV absorption spectra of calmodulin prepared from control and MHS porcine brain were virtually identical.

3.3.4 Functional Studies of Calmodulin Isolated from Control and Malignant Hyperpyrexia Susceptible Porcine Brain

3.3.4.1 Activation of Phosphodiesterase

The most commonly used assay of the activity of calmodulin is its ability to activate a calmodulin-dependent 3':5'-cyclic nucleotide phosphodiesterase (Teo, Wang and Wang, 1973). This enzyme degrades cyclic-AMP to 5'-AMP, which can be further degraded by 5'-nucleotidase to release inorganic phosphate (P_i). The amount of P_i released is measured photometrically.

The abilities of calmodulins isolated from control and MHS porcine brain to activate phosphodiesterase were similar (Figure 3.6). They were also similar to published activities for calmodulin isolated from bovine brain (Lin, Liu and Cheung, 1974; Sharma and Wang, 1979). Fifty per cent activation of 0.016 units of phosphodiesterase was achieved using 30-40 ng of calmodulin.

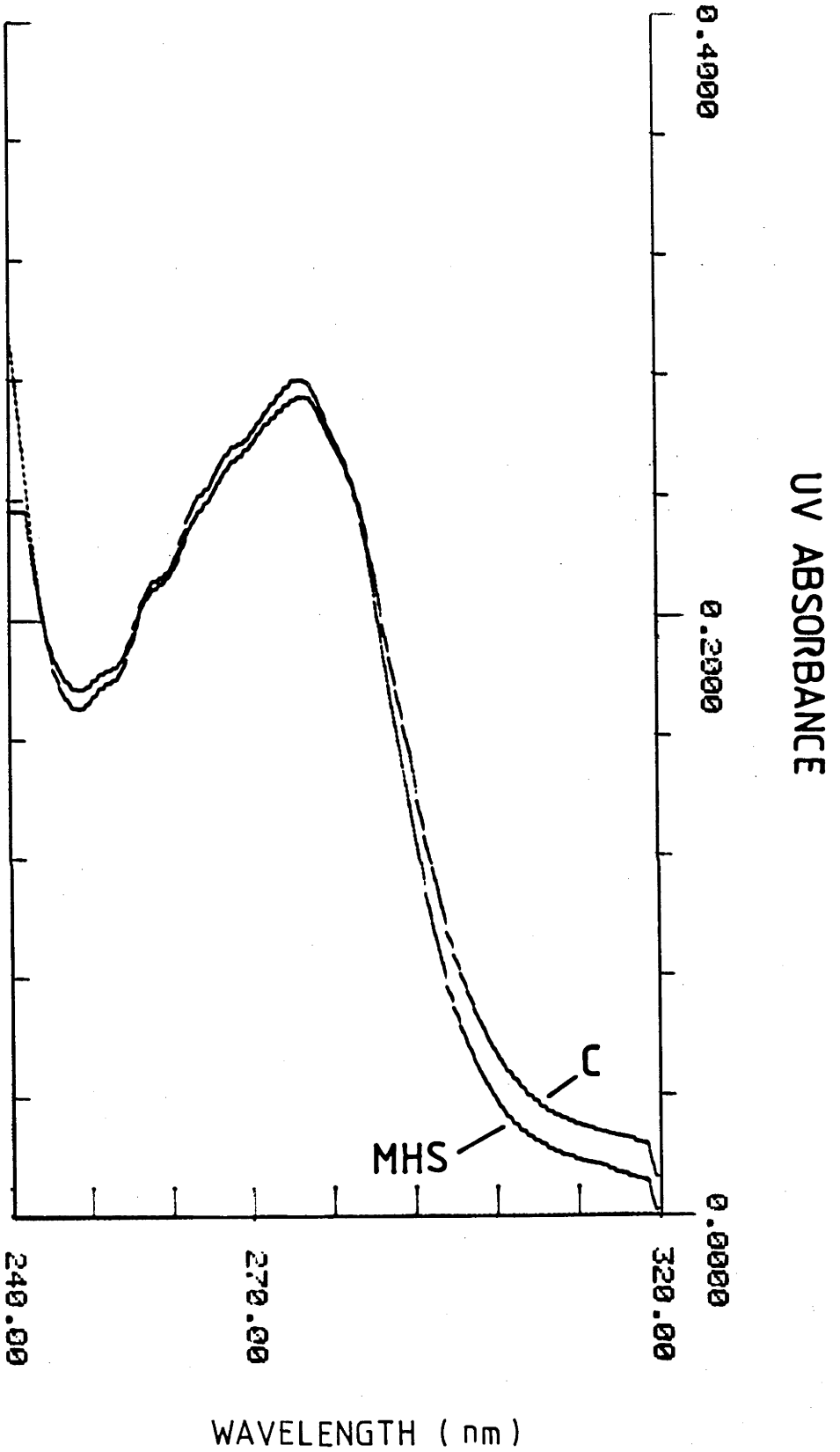


Figure 3.5 Comparison of the Ultra-violet Absorbance Spectra of Calmodulin Isolated from Control and Malignant Hyperpyrexia Susceptible Porcine Brain.

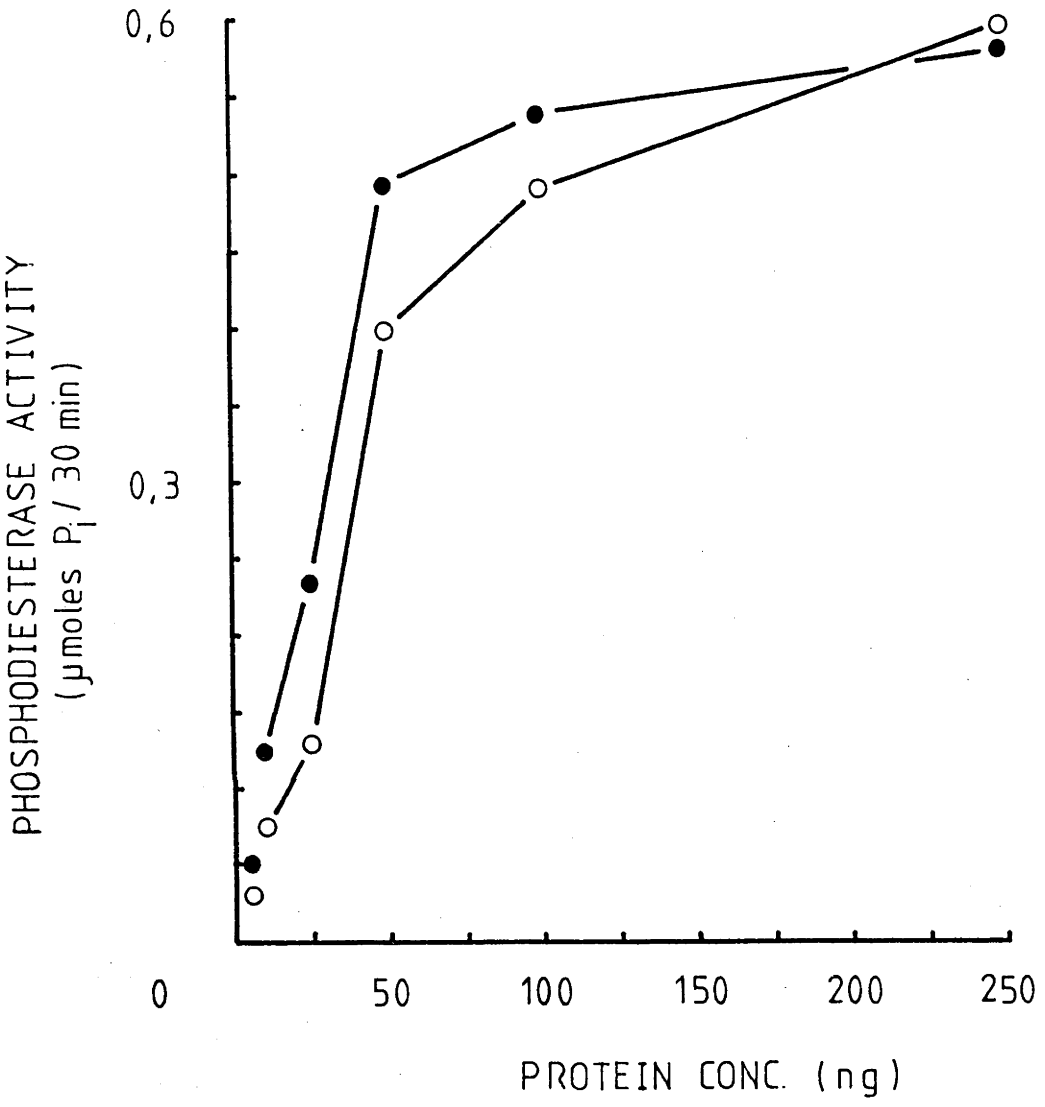


Figure 3.6 The Activation of Phosphodiesterase by Control and Malignant Hyperpyrexia Susceptible Porcine Brain Calmodulin.

o Control

• MHS

3.3.4.2 Inhibition of Calmodulin-Activated Phosphodiesterase by Calmodulin Antagonists

Calmodulins isolated from control and MHS porcine brain were also compared by investigating the pattern of inhibition of phosphodiesterase, activated by these calmodulins, by calmodulin antagonists. Trifluoperazine and HPD were chosen for these investigations because of their different inhibitory potencies (Table 2.1) and the characteristics of their *in vitro* skeletal muscle pharmacology. Both TFP and HPD were able to produce contracture in control and MHS muscle, but HPD contractures were reversed by dantrolene sodium to a significantly greater extent than TFP contractures (Table 2.3). The effect of dantrolene sodium on the inhibition of calmodulin-activated phosphodiesterase activity by TFP and HPD was investigated because dantrolene sodium reversed contractures induced *in vitro* by calmodulin antagonists and this reversal might be due to a direct effect of dantrolene sodium on calmodulin.

Phosphodiesterase activities activated by calmodulins isolated from control and MHS porcine brain were inhibited by TFP to a similar degree (Figures 3.7 and 3.8). An I_{50} concentration for TFP of approximately 12 μM was observed for both control and MHS calmodulin-activated activities. Dantrolene sodium had no effect on the inhibition by TFP of the phosphodiesterase activities activated by control or MHS calmodulin.

Phosphodiesterase activities activated by calmodulins isolated from control and MHS porcine brains were inhibited by HPD to a similar

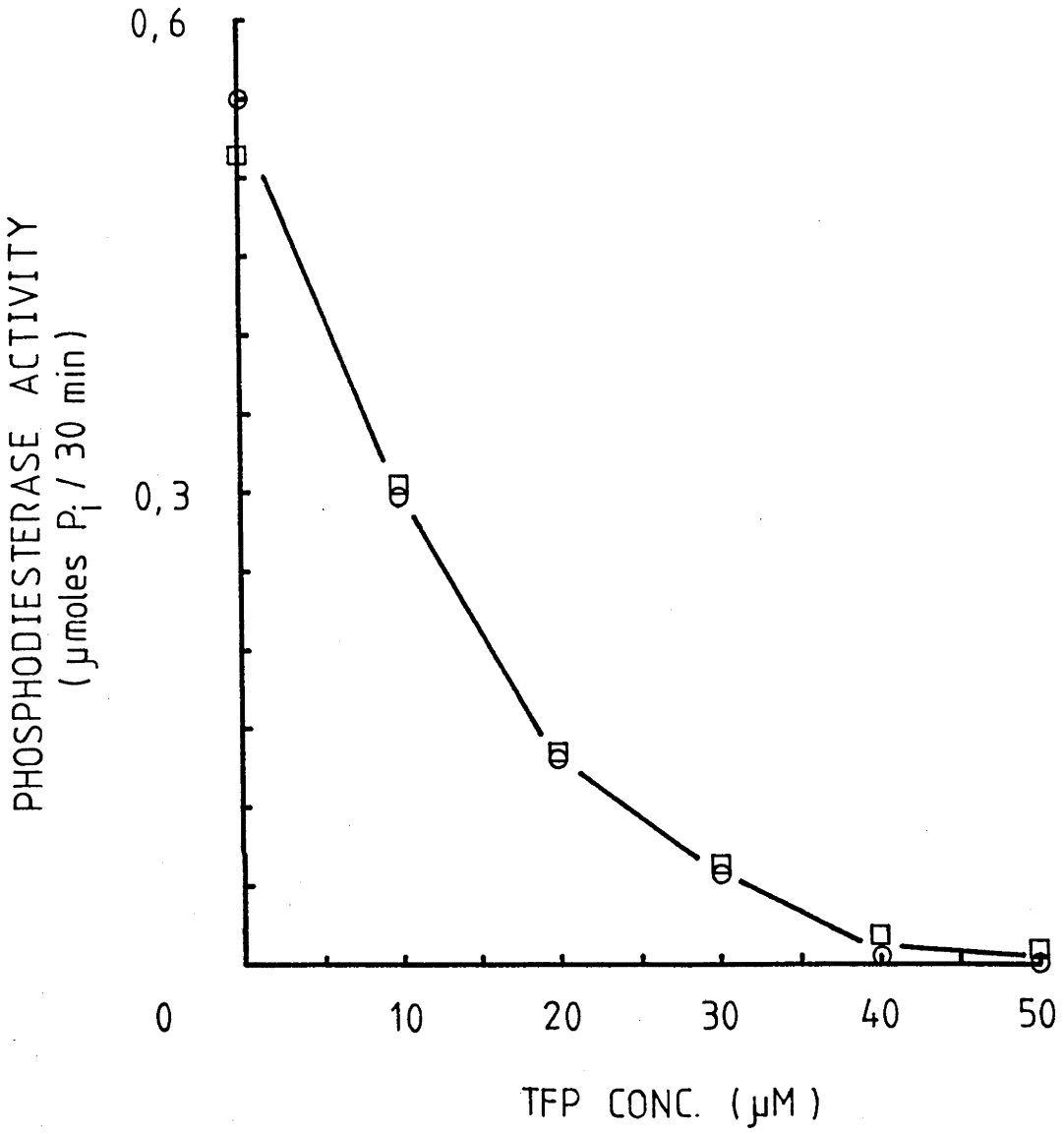


Figure 3.7 The Inhibition by Trifluoperazine of Phosphodiesterase Activity Activated by Control Porcine Brain Calmodulin

o TFP

□ TFP plus 20 μM dantrolene sodium

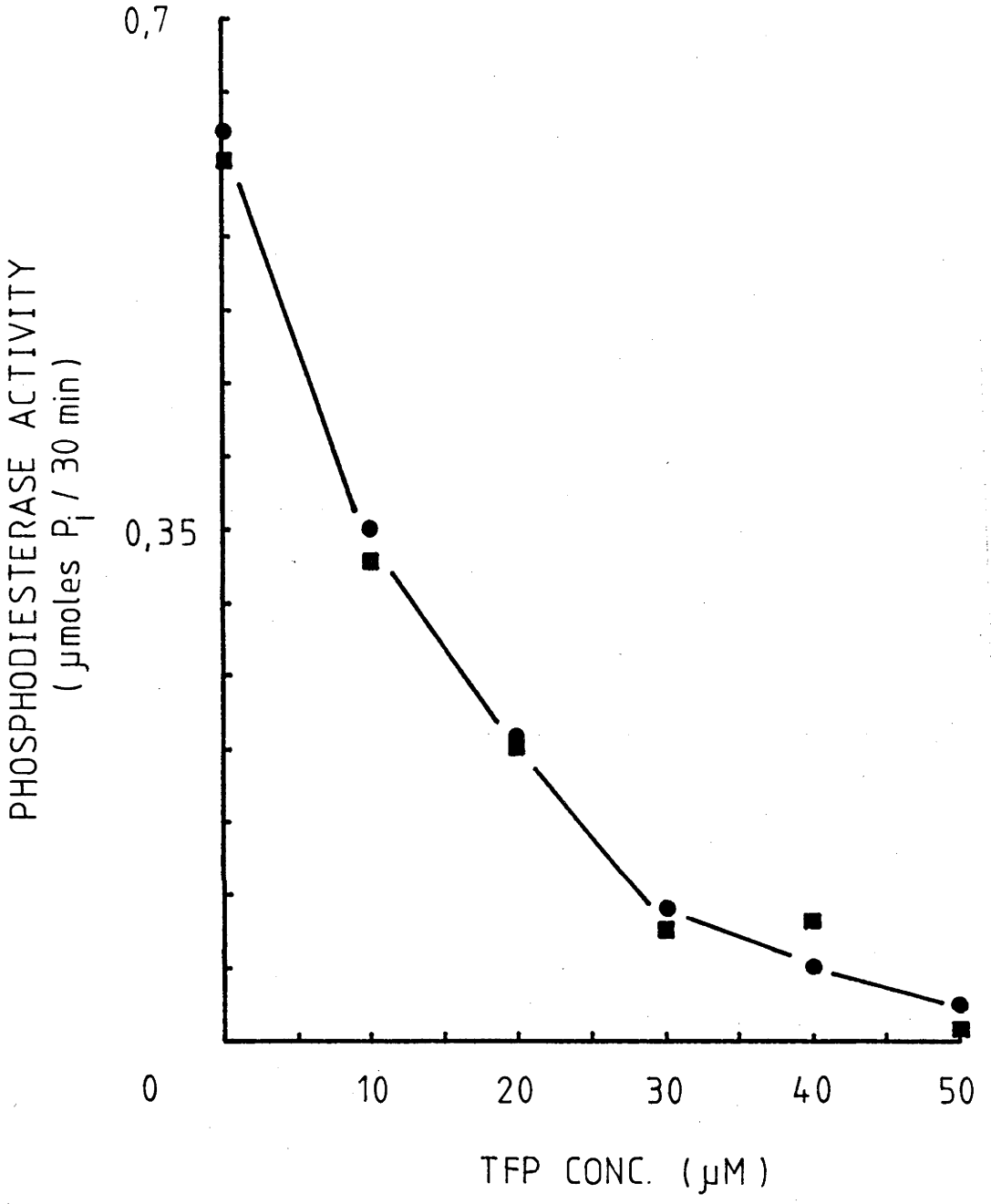


Figure 3.8 The Inhibition by Trifluoperazine of Phosphodiesterase Activity Activated by Malignant Hyperpyrexia Susceptible Porcine Brain Calmodulin

● TFP

■ TFP plus 20 µM dantrolene sodium

degree (Figures 3.9 and 3.10). An I_{50} concentration for HPD of approximately 500 μM was observed for both control and MHS calmodulin-activated activities. Dantrolene sodium had no effect on the inhibition by HPD of the phosphodiesterase activities activated by control or MHS calmodulin.

(The results of the phosphodiesterase activation and drug inhibition studies of calmodulin were confirmed using another control and MHS preparation.)

3.3.5 The Concentrations of Cyclic-Adenosine Monophosphate in Control and Malignant Hyperpyrexia Susceptible Porcine Skeletal Muscle

Calmodulin has been shown to regulate the activities of the enzymes phosphodiesterase and adenyl cyclase in a number of tissues (Cheung, Lynch and Wallace, 1978; Smoake, Song and Cheung, 1974). Thus, the activity and levels of calmodulin in a specific tissue may regulate the concentration of cyclic-AMP in that tissue. Because of this, the concentration of cyclic-AMP in control and MHS skeletal muscle was investigated as a possible indicator of calmodulin function in these tissues.

Biopsy specimens from the gracilis muscle of control and MHS swine were analysed for their cyclic-AMP concentration using a commercially available cyclic-AMP test kit. Control muscle was found to contain 213 ± 67 pmoles of cyclic-AMP per gram of tissue and 9.6 ± 4.8 pmoles of cyclic-AMP per mg of protein (mean ± S.D.). Muscle from MHS swine

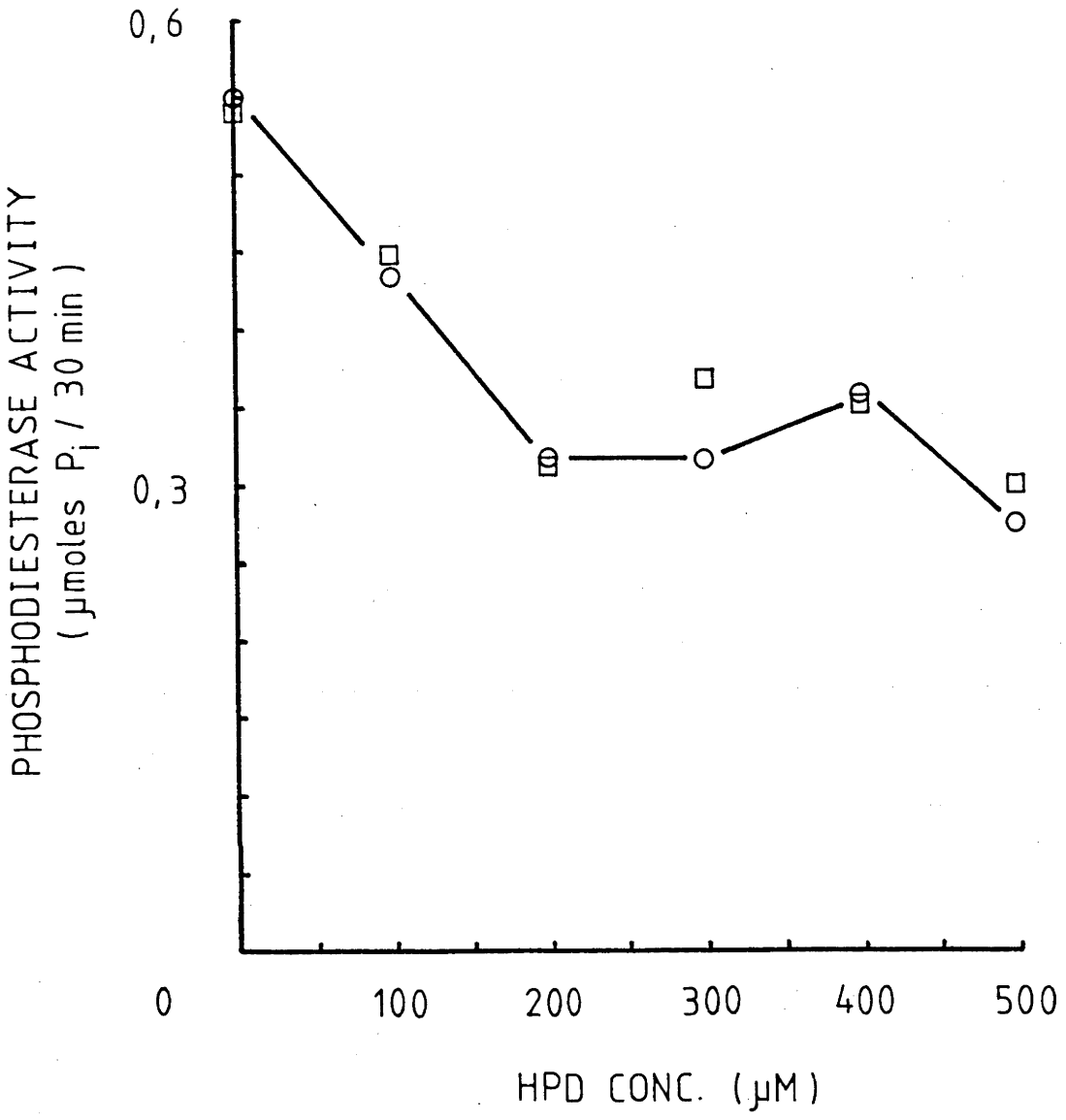


Figure 3.9 The Inhibition by Haloperidol of Phosphodiesterase Activity Activated by Control Porcine Brain Calmodulin

o HPD

□ HPD plus 20 µM dantrolene sodium

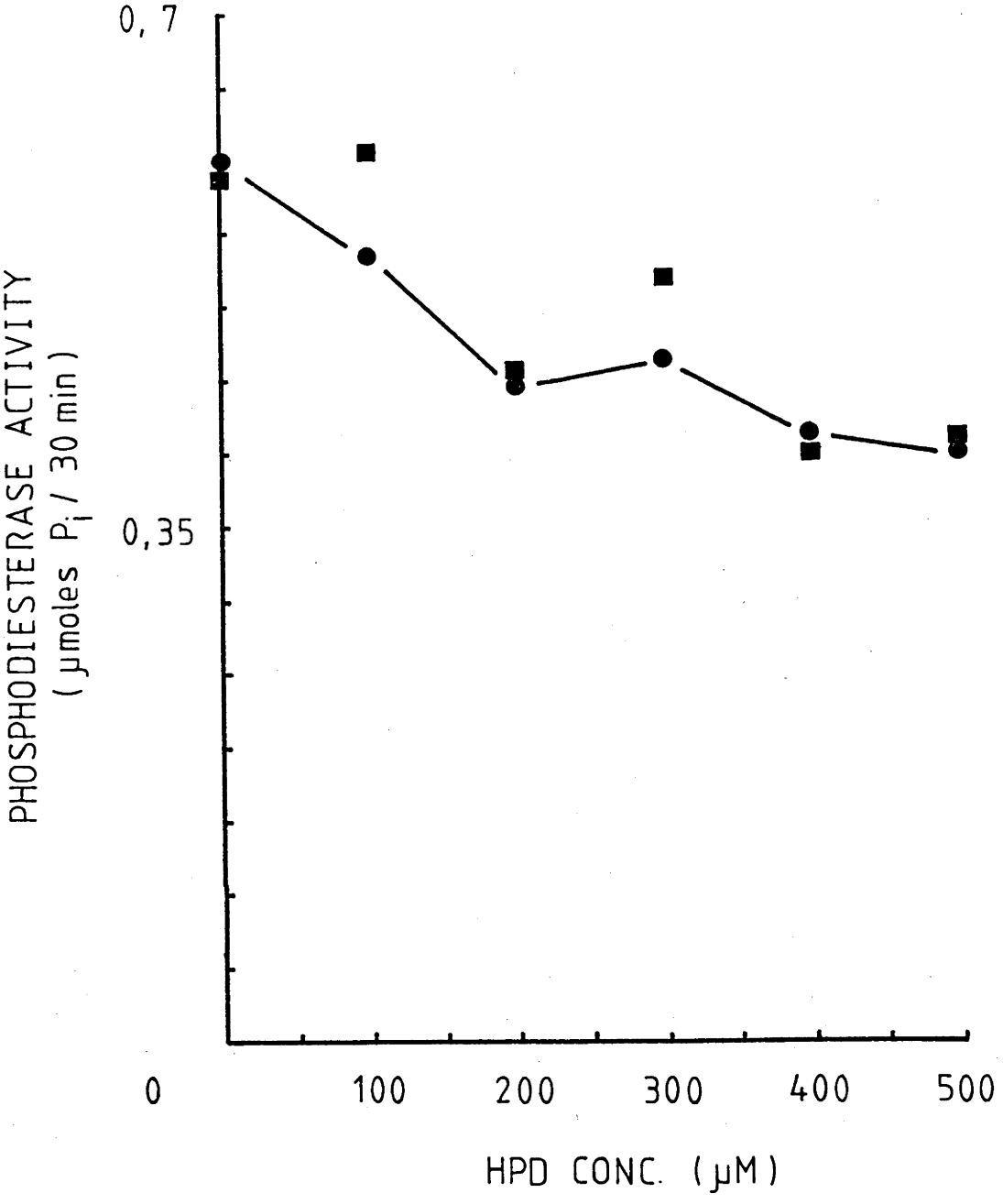


Figure 3.10 The Inhibition by Haloperidol of Phosphodiesterase Activity Activated by Malignant Hyperpyrexia Susceptible Porcine Brain Calmodulin

- ● HPD
- ■ HPD plus 20 µM dantrolene sodium

was found to contain 225 ± 60 pmoles of cyclic-AMP per gram of tissue and 7.2 ± 2.5 pmoles of cyclic-AMP per mg of protein (mean + S.D.) (Table 3.1). These concentrations did not differ significantly between control and MHS muscle.

3.4 Discussion

The results obtained in this chapter showed that the calmodulins isolated from control and MHS porcine brain did not differ in their physical or functional characteristics. Because of the reported lack of tissue specificity of calmodulin (Wang and Waisman, 1979; Kakiuchi, Sobue, Yamazaki, Kambayashi, Sakon and Kosaki, 1981) the isolated brain calmodulin was assumed to be identical to the calmodulin present in the skeletal muscle of control and MHS swine. The procedure for the isolation of calmodulin used in the present investigation employed an affinity chromatography step for the final purification (Kakiuchi, Sobue, Yamazaki, Kambayashi, Sakon and Kosaki, 1981). After the porcine brain tissue had been homogenized, extracted, and the extract subjected to ion exchange chromatography, the fractions containing calmodulin activity were applied to a Sepharose 6B-coupled fluphenazine affinity column. The initial conditions of the affinity column, that is, the presence of Ca^{2+}, allowed calmodulin to bind to the immobilized fluphenazine. Replacement of the Ca^{2+} present in the elution buffer with EGTA allowed the calmodulin to be eluted from the affinity column. The calmodulins from both control and MHS porcine brain behaved identically throughout the isolation procedure.

Table 3.1 **The Concentration of Cyclic-Adenosine Monophosphate in Control and Malignant Hyperpyrexia Susceptible Porcine Skeletal Muscle.**

	Cyclic-AMP concentration (mean ± S.D.)		n
	pmoles/g tissue	pmoles/mg protein	
Control	213 ± 67	9.6 ± 4.8	10
MHS	225 ± 60	7.2 ± 2.5	21

The polyacrylamide gel patterns of the calmodulin preparations isolated from control and MHS porcine brain did not differ. This observation is consistent with a report by Lorkin and Lehmann (1983b). These authors, using 2 dimensional electrophoresis, found no differences between calmodulins isolated from control and MHS porcine erythrocytes. In the present study, the MW of the control and MHS calmodulins were identical to the MW of a commercially available porcine brain calmodulin, and comparable to the MW of calmodulins isolated from various sources (approximately 17,000 MW) (Cheung, 1980; Scharff, 1981). Three protein contaminants in the MW range 19,000 to 21,000 are present in the control, MHS and commercial calmodulin preparations. Since the commercial calmodulin preparation was isolated by an affinity chromatography procedure similar to the one employed in the present investigation, these contaminants appear to be brain proteins which, like calmodulin, had the ability to bind to the affinity column in a Ca^{2+}-dependent manner. The low MW contaminants (approximately 14,000 MW) which were present in the control and MHS calmodulin preparations, but not the commercial calmodulin preparation, may have been breakdown products of calmodulin formed after prolonged storage.

The UV absorption spectra (240 nm to 320 nm) of calmodulins isolated from control and MHS porcine brain were virtually identical. Calmodulin had a distinctive UV absorption pattern because of its high ratio of phenylalanine (eight residues) to tyrosine (two residues) (Cheung, 1980). This type of UV absorbance spectrum was similar to that of another Ca^{2+}-binding protein, troponin C, which

shares significant sequence homology with calmodulin (Watterson, Harrelson, Keller, Sharief and Vanaman, 1976).

Functional studies of calmodulins isolated from control and MHS porcine brain did not detect any differences in the function of these proteins. The assay of calmodulin function used in the present investigation was the activation of a calmodulin-deficient phosphodiesterase from bovine heart. The activation curves of control and MHS calmodulin preparations were similar with 50% activation of phosphodiesterase requiring 30 - 40 ng of calmodulin. This 50% activation figure was comparable to that obtained for bovine brain calmodulin (Lin, Liu and Cheung, 1974; Sharma and Wang, 1979) and confirmed an earlier study of control and MHS porcine brain calmodulin activation of phosphodiesterase (Marjanen, Collins and Denborough, 1984). The ability of calmodulin antagonist drugs to inhibit calmodulin-activated phosphodiesterase activity was also used to compare the control and MHS calmodulin preparations. The drugs TFP and HPD inhibited calmodulin-activated phosphodiesterase activity activated by control and MHS calmodulins, and the characteristics and extent of this inhibition did not differ between these calmodulins. These results using TFP were consistent with an earlier report using control and MHS porcine brain calmodulin (Marjanen, Collins and Denborough, 1984). The I_{50} concentration for TFP inhibition of phosphodiesterase activity activated by control and MHS brain calmodulin of 12 μM obtained in the present study is comparable to a published TFP I_{50} value of 10 μM using bovine brain calmodulin (Levin and Weiss, 1976). The I_{50} concentration for HPD inhibition of phosphodiesterase activity activated by control and MHS brain

calmodulin of approximately 500 μM was much higher than a published HPD I_{50} value of 60 μM (Levin and Weiss, 1979). Levin and Weiss (1979) used calmodulin from bovine brain, a phosphodiesterase from rat brain and a luciferin-luciferase method to determine phosphodiesterase activity in their HPD I_{50} study, but the reason for this HPD I_{50} discrepancy is not apparent.

The effect of the skeletal muscle relaxant dantrolene sodium on the inhibition of calmodulin function by calmodulin antagonist drugs was investigated because of the ability of dantrolene sodium to partially reverse contractures induced by calmodulin antagonist drugs in control and MHS skeletal muscle in vitro (Chapter 2.3.3). However dantrolene sodium had no effect on the inhibition of calmodulin-activated phosphodiesterase activity by TFP or HPD, using either control or MHS porcine brain calmodulin. This observation suggested the reversal of calmodulin antagonist drug-induced contractures by dantrolene sodium was not due to a direct interaction of dantrolene sodium and calmodulin. It also implied that calmodulin antagonist drugs acted in vitro at a site other than calmodulin which was sensitive to dantrolene sodium. Dantrolene sodium is thought to act on E-C coupling in skeletal muscle (Ellis and Bryant, 1972) and, therefore, the calmodulin antagonist drugs may have acted in, part, on E-C coupling to induce in vitro contractures in control and MHS skeletal muscle.

Since calmodulin has been shown to regulate the activities of the enzymes phosphodiesterase and adenyl cyclase in a number of tissues (Smoake, Song and Cheung, 1974; Cheung, Lynch and Wallace, 1978), the

concentration of cyclic-AMP in control and MHS porcine skeletal muscle was investigated as a possible indirect indicator of calmodulin function in these tissues. This indicator was based on two assumptions. Firstly, that there were calmodulin-dependent forms of phosphodiesterase and adenyl cyclase present in porcine skeletal muscle, and secondly, that the proportion of these calmodulin-dependent forms were high enough compared to calmodulin-independent forms to influence cyclic-AMP levels. An increase in the content of cyclic-AMP has been reported for human MHS muscle when compared to control muscle (Willner, Cerri and Wood, 1981). An increased concentration of cyclic-AMP has also been reported in MHS swine longissimus muscle at three minutes post mortem (Ono, Topel and Althen, 1976), but no changes in the activities of phosphodiesterase or adenyl cyclase were detected (Ono, Topel and Althen, 1977). In the present investigation, no significant difference was found between the concentrations of cyclic-AMP in control and MHS gracilis muscle. Taking into account the assumptions mentioned above, this observation implied that there was no detectable difference in the function of calmodulin in control and MHS skeletal muscle.

3.5 Summary

The effects of the calmodulin antagonist drugs on the contractile characteristics of control and MHS porcine skeletal muscle *in vitro* prompted a biochemical investigation of the Ca^{2+}-regulatory protein calmodulin isolated from control and MHS porcine brain. The physical properties of these two proteins, that is their behaviour during the isolation procedure, their characteristics on polyacrylamide gel

electrophoresis and their UV absorbance spectra, did not differ. The functional properties of calmodulins isolated from control and MHS brain, that is their activation of the enzyme phosphodiesterase and the characteristics of the inhibition of this activity by calmodulin antagonist drugs, were similar. Also, the concentration of cyclic-AMP in the gracilis muscle of control and MHS swine, which was investigated as a possible indicator of calmodulin function in these tissues, was not significantly different. These observations suggested that MH in swine is not due to an abnormality in calmodulin.

CHAPTER 4 THE EFFECTS OF CALMODULIN ANTAGONISTS ON ISOLATED SARCOPLASMIC RETICULUM FROM CONTROL AND MALIGNANT HYPERPYREXIA SUSCEPTIBLE SWINE

4.1 Introduction

In the preceding chapters the effects of calmodulin antagonists on skeletal muscle from control and MHS swine have been described. In vitro pharmacological observations (Chapter 2) showed that these drugs caused control swine skeletal muscle to behave in a similar manner to muscle from MHS swine, that is, they induced hypercontractility to halothane, caffeine and succinylcholine. Dantrolene sodium partially reversed these contractures. In addition, the hypercontractility of MHS muscle was potentiated by calmodulin antagonists. Calmodulins isolated from control and MHS porcine brain were indistinguishable (Chapter 3). Trifluoperazine was shown to inhibit both control and MHS brain calmodulin with an I_{50} concentration of 12 μM. Haloperidol, which was at least as potent as TFP in the in vitro pharmacology, was shown to inhibit both control and MHS brain calmodulin with an I_{50} concentration of 500 μM. Observations such as these suggested that calmodulin antagonist drugs may have been acting at sites other than calmodulin to induce their effects on the contractile characteristics of skeletal muscle.

Balzer, Makinose and Hasselbach (1968) found that CPZ inhibited active Ca^{2+} uptake by isolated SR. Andersson (1972) and Takagi (1981) both concluded that the major contractile effects produced by CPZ in skeletal muscle fibres were mediated by the release of Ca^{2+} from the SR by this drug. Although calmodulin does not appear to be involved in the regulation of the skeletal muscle SR Ca^{2+}-ATPase

(Chiesi and Carafoli, 1982), it may be involved in the regulation of Ca^{2+} release from the SR (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1983).

In this chapter investigations will be described which were designed to investigate the effect of calmodulin antagonists on isolated SR membranes in relation to MH. These included the effects of calmodulin antagonists on Ca^{2+}-ATPase activity, ATP-dependent Ca^{2+} uptake and Ca^{2+} efflux.

4.2 Materials and Methods

4.2.1 Drugs and Reagents

The following gifts of drugs are gratefully acknowledged: TFP from Smith, Kline and French Laboratories Australia; tritiated TFP from Smith, Kline and French Research Laboratories, Hertfordshire, England; PRO and CPZ from May and Baker Australia Pty Ltd; FPZ from E.R. Squibb and Sons; HPD from Searle Laboratories; PEN and PIM from Janssen Pharmaceutica, N.V., Belgium; and dantrolene sodium from Norwich Pharmaceuticals.

R24571 was obtained from Boehringer Mannheim, GmbH.

All other reagents were of analytical grade.

4.2.2 Preparation of the Sarcoplasmic Reticulum Vesicles

Femoris muscle was removed from either control or MHS swine immediately post-mortem, ground and frozen in liquid nitrogen. It was stored at -70^{o}C until used.

The frozen ground muscle was allowed to thaw in ice-cold 0.25 M sucrose, 10 mM Tris (pH 7.4) (Tris-sucrose) before being homogenized in a Waring blender (2x30 second homogenizations at low speed). All subsequent steps were carried out between 0^{o} and 4^{o}C. The homogenate was filtered through gauze and centrifuged at 3000xg for 5 minutes to remove myofibrils and connective tissue. The supernatant was centrifuged at 10,000xg for 20 minutes to remove mitochondria. The resulting supernatant was centrifuged at 100,000xg for 20 minutes to pellet the membranes. This pellet was washed with the same Tris-sucrose buffer and the resulting pellet was resuspended in this buffer. This preparation was referred to as the fragmented sarcoplasmic reticulum, (FSR) (Lau, Caswell and Brunschwig, 1977; White, Collins and Denborough, 1983).

4.2.3 Preparation of Ethyleneglycol-bis-(β-amino-ethylether)-N, N'-tetraacetate Washed Sarcoplasmic Reticulum Vesicles

Fresh FSR preparations were resuspended in 0.25 M sucrose, 10 mM Tris (pH 8.0), 1 mM histidine and 1 mM ethyleneglycol-bis-(β-aminoethylether)-N, N'-tetraacetate (EGTA) and allowed to stand in ice for 15 minutes. The membranes were pelleted at 100,000xg for 20 minutes and resuspended in Tris-sucrose buffer.

4.2.4 The Calcium-Dependent Adenosine Triphosphatase Assays

Method a. The reaction buffer consisted of 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM histidine (pH 6.8), 5 mM oxalate and 1 mM ATP. 0.5 ml of this buffer was pipetted into test tubes standing in a water-ice slurry. To the appropriate tubes were added SR suspension (20-40 μg protein), and 0.5 mM $CaCl_2$ or 1 mM EGTA. The reaction was started by immersing the tubes in a 37^{o}C water bath. After 10 minutes

the reaction was stopped by the addition of 4 ml of inorganic phosphate reagent. The Ca^{2+}-dependent ATPase activity was calculated as the difference between the activity in the presence of added Ca^{2+} and the activity in the presence of EGTA.

Method b. The procedure followed in this method was the same as for method a. except that the reaction buffer consisted of 100 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 50 μM EGTA, 1 mM ATP and 10 mM Tris (pH 7.4). Also, $CaCl_2$ was added at 10 μM, 20 μM, 30 μM and 40 μM to a series of tubes to produce a Ca^{2+}-dependent increase in activity.

4.2.5 The Adenosine Triphosphate-Dependent Calcium Uptake Assay

The reaction buffer consisted of 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris (pH 7.4), 5 mM oxalate and 20 μM $CaCl_2$. To 0.45 ml of this buffer at 37°C were added $^{45}Ca^{2+}$ and SR vesicle suspension (75-100 μg protein). The reaction was started by the addition of 1 mM ATP. 50 μl samples were withdrawn from the reaction medium at 15 seconds, 30 seconds, 60 seconds and 120 seconds and filtered with 2 x 3 ml of ice cold reaction buffer on an Amicon filtration manifold. The filters used were of 0.22 μm pore size. After filtration the filters were dissolved in 10 ml of scintillation fluid and the radioactivity present was counted in a liquid scintillation counter.

4.2.6 Passively Loaded Sarcoplasmic Reticulum Calcium Efflux Assay

The reaction buffer consisted of 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris (pH 7.4) and 1 mM $CaCl_2$. Passive loading took place overnight between 0° and 4°C in a suspension of SR vesicles (1.5-3 mg protein in 1 ml of reaction buffer) in the presence of $^{45}Ca^{2+}$. Efflux experiments were started by adding 50 μl of the preloaded vesicles to

1.95 ml of reaction buffer at 37°C. 200 µl samples were withdrawn at 0, 0.5, 1, 2, 5 and 10 minutes and were filtered with 2x3 ml of ice-cold reaction buffer on an Amicon filtration manifold. Test drugs were added at t = 15 seconds. The filters used were of 22 µm pore size. After filtration the filters were dissolved in 10 ml of scintillation fluid and the radioactivity present was counted in a liquid scintillation counter.

4.2.7 Ultraviolet Light-Activated Binding of Tritiated Trifluoperazine to Sarcoplasmic Reticulum Membrane Proteins

Samples of SR vesicles (1.5-3 mg protein) in 1 ml of 5 mM Tris (pH 7.0), 1 mM $MgCl_2$ were irradiated with UV light in the presence of ^{3}H-TFP (100-500 µM) and 500 µM Ca^{2+} for up to 90 minutes in an icebath with stirring. In some experiments a pre-irradiation in the presence of unlabelled TFP and 0.5 mM EGTA was included. Binding was assessed by separating the SR membrane proteins by polyacrylamide gel electrophoresis on 7.5% to 15% acrylamide gradient slab gels,slicing the gels into sections, and counting the radioactivity associated with each slice. Gel slices were dissolved in 30% H_2O_2 overnight at 60°C. 10 ml of scintillation fluid was added and the radioactivity was counted in a liquid scintillation counter.

4.2.8 Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was performed according to the method of Laemmli (1970) using 7.5% to 15% acrylamide gradient slab gels with 4.5% acrylamide stacking gels. Gels were run at 40 ma/gel and stained with Coomassie blue.

4.2.9 Inorganic Phosphate Determinations

Inorganic phosphate was determined according to the method of Heinonen and Lahti (1981).

4.2.10 Protein Determinations

Protein determinations were performed using a modification of the Lowry method (Peterson, 1977).

4.2.11 Statistical Methods

Data was analysed by Student's t-test according to Hinchen (1969) or using the computer-based "Statistical Package for the Social Sciences" (Nie, Hull, Jenkins, Steinbrenner and Bent, 1970).

4.3 Results

4.3.1 Fragmented Sarcoplasmic Reticulum

The Ca^{2+}-dependent ATPase activities of FSR preparations isolated from control and MHS porcine skeletal muscle did not differ significantly (Table 4.1) and were comparable to values reported by McIntosh, Berman and Kench (1977). Contamination of FSR preparations from our laboratory by mitochondria, lysosomes and sarcolemma have been shown to be low (White, Collins and Denborough, 1983).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) patterns showed similar protein compositions for both control and MHS FSR preparations (Figures 4.1 and 4.2). The major protein bands in these gels correspond to the Ca^{2+}-dependent ATPase (approximately 100,000 MW) and the calcium binding protein calsequestrin (approximately 66,000 MW) (White, Thomas and Denborough, 1983). A number of bands appear in the region of the MW of calmodulin

Table 4.1 Calcium-Dependent Adenosine Triphosphatase Activities of Fragmented Sarcoplasmic Reticulum Preparations Isolated from Control and Malignant Hyperpyrexia Susceptible Porcine Skeletal Muscle

	ATPase Activity (μmole P_i per minute per mg protein, mean ± S.E.)	n
Control	1.18 ± 0.1	15
MHS	1.24 ± 0.07	9

(approximately 17,000; Watterson, Sharief and Vanaman, 1980). Calmodulin was unlikely to be visible on this gel, however. Published estimates of the amount of calmodulin present in isolated SR preparations (Chiesi and Carafoli, 1982) indicate that approximately 50 ng of this protein was applied to the gel which is below the amount required to be detected by the staining technique used here.

4.3.2 The Effect of Trifluoperazine on the Ca^{2+}-Dependent Adenosine Triphosphatase Activity of Fragmented Sarcoplasmic Reticulum

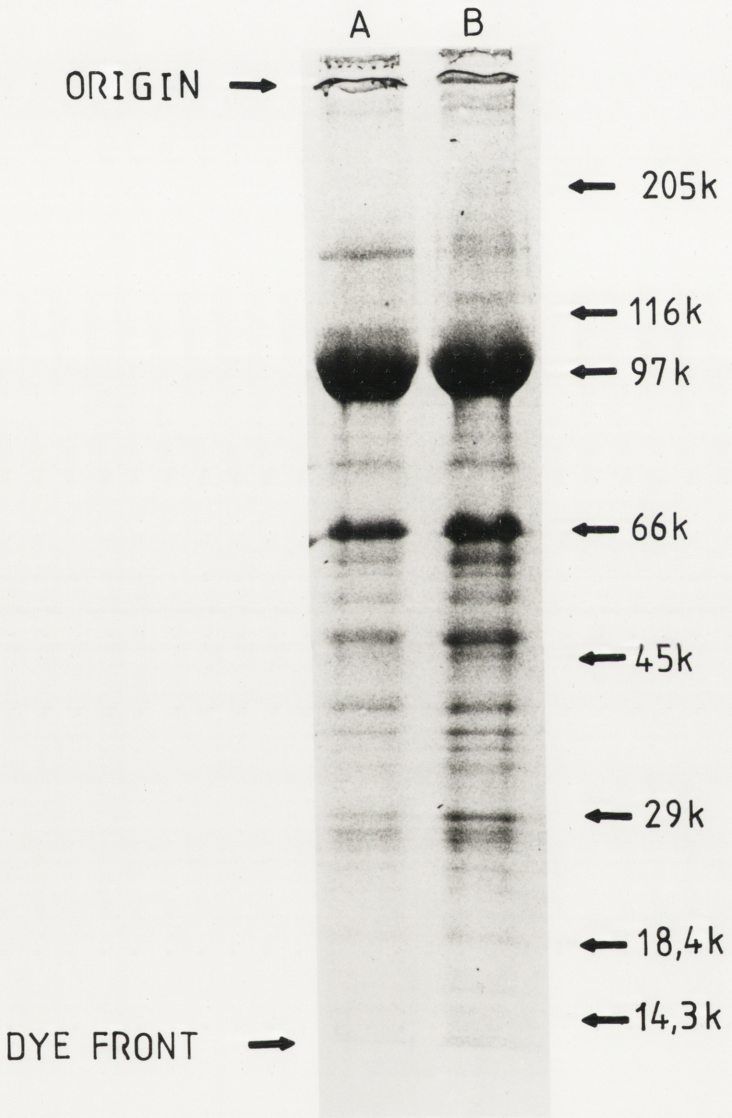
Trifluoperazine inhibited the Ca^{2+}-dependent ATPase activities of both control and MHS FSR with an I_{50} concentration of approximately 250 μM (Figures 4.3 and 4.4). The FSR ATPase activity was almost completely abolished at a TFP concentration of 500 μM. Washing the vesicles with a solution containing EGTA to remove calmodulin associated with the outer surface of the vesicles had no effect on the extent of inhibition of Ca^{2+}-dependent ATPase activity by TFP in either control or MHS FSR (Table 4.2).

The effect of dantrolene sodium on the inhibition of control and MHS FSR Ca^{2+}-dependent ATPase activity by TFP was investigated using the Ca^{2+}-ATPase assay method b (Materials and Methods 4.2.4). This method used a fixed concentration of TFP (150 μM) to achieve approximately 50% inhibition of Ca^{2+}-ATPase activity over a range of Ca^{2+} concentrations (zero to 40 μM). It was considered that this method would have been more sensitive in detecting any dantrolene sodium reversal of TFP inhibition than method a. which used a high Ca^{2+} concentration. Dantrolene sodium had no effect on the

Figure 4.1 Polyacrylamide Gel Patterns of Fragmented Sarcoplasmic Reticulum Preparations Isolated from Malignant Hyperpyrexia Susceptible and Control Porcine Skeletal Muscle.

A. MHS

B. Control


A
B
ORIGIN
205k
116k
97k
66k
45k
29k
18,4k
14,3k
DYE FRONT

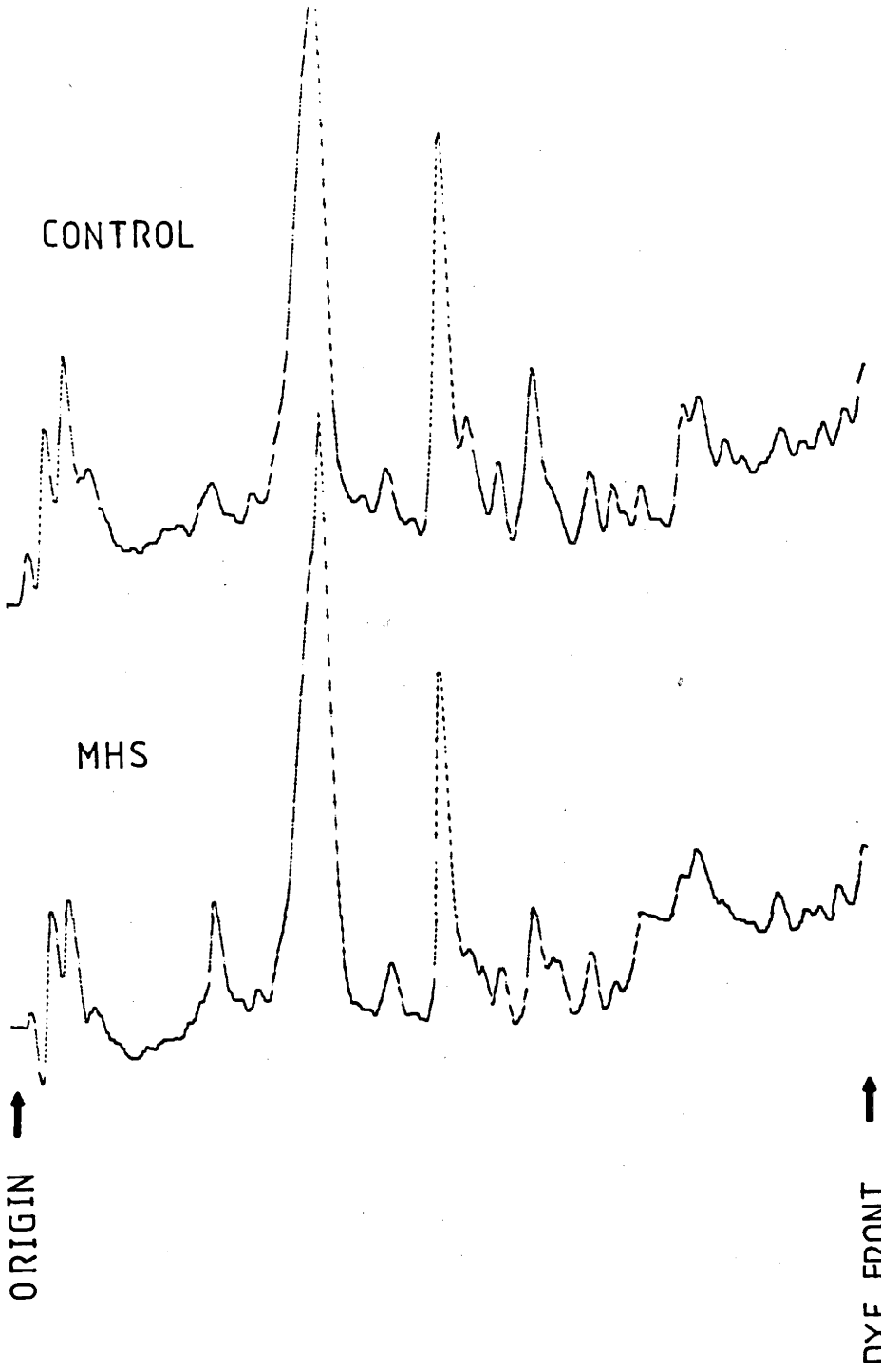


Figure 4.2 Comparison of Absorbance Scans of Polyacrylamide Gels of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum Preparations.
Wavelength = 540 nm.

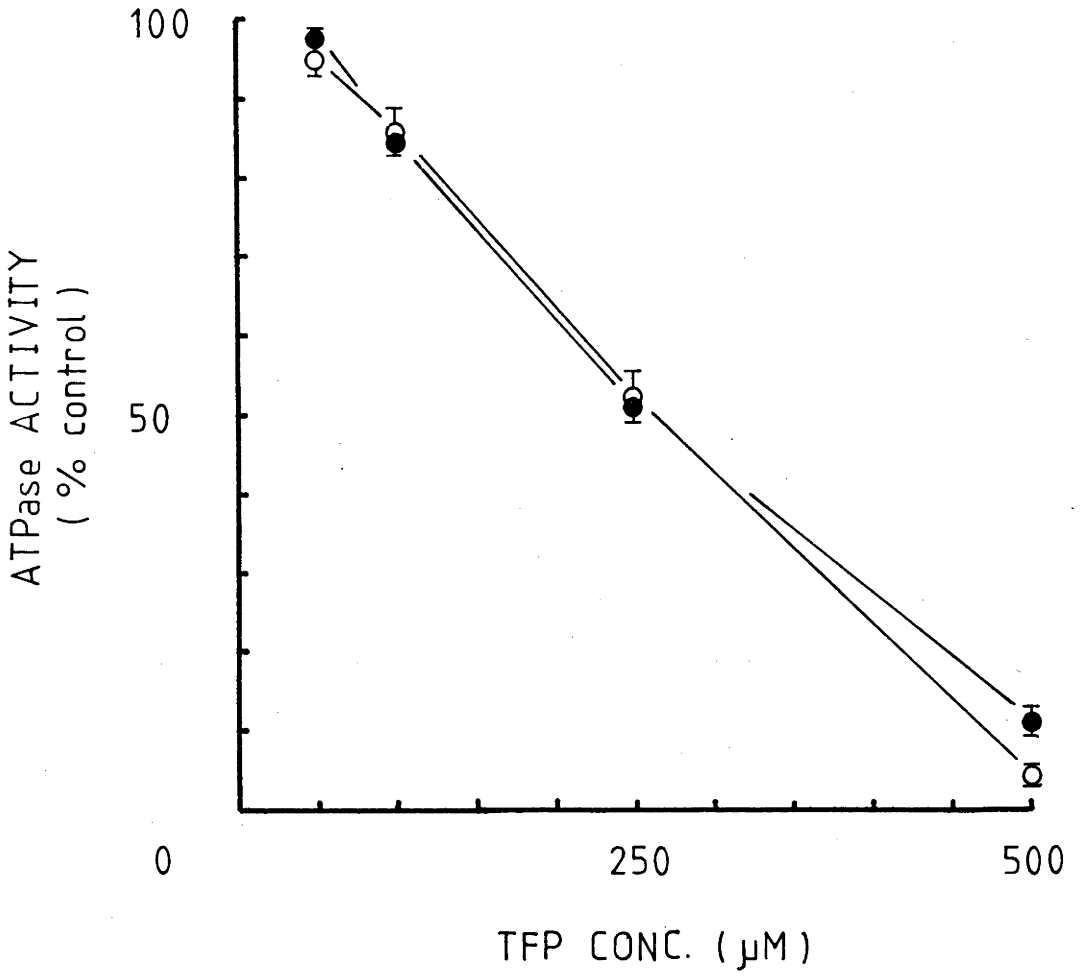


Figure 4.3 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Trifluoperazine.

o Control

• MHS

Each value is the mean ± S.E. of 4 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

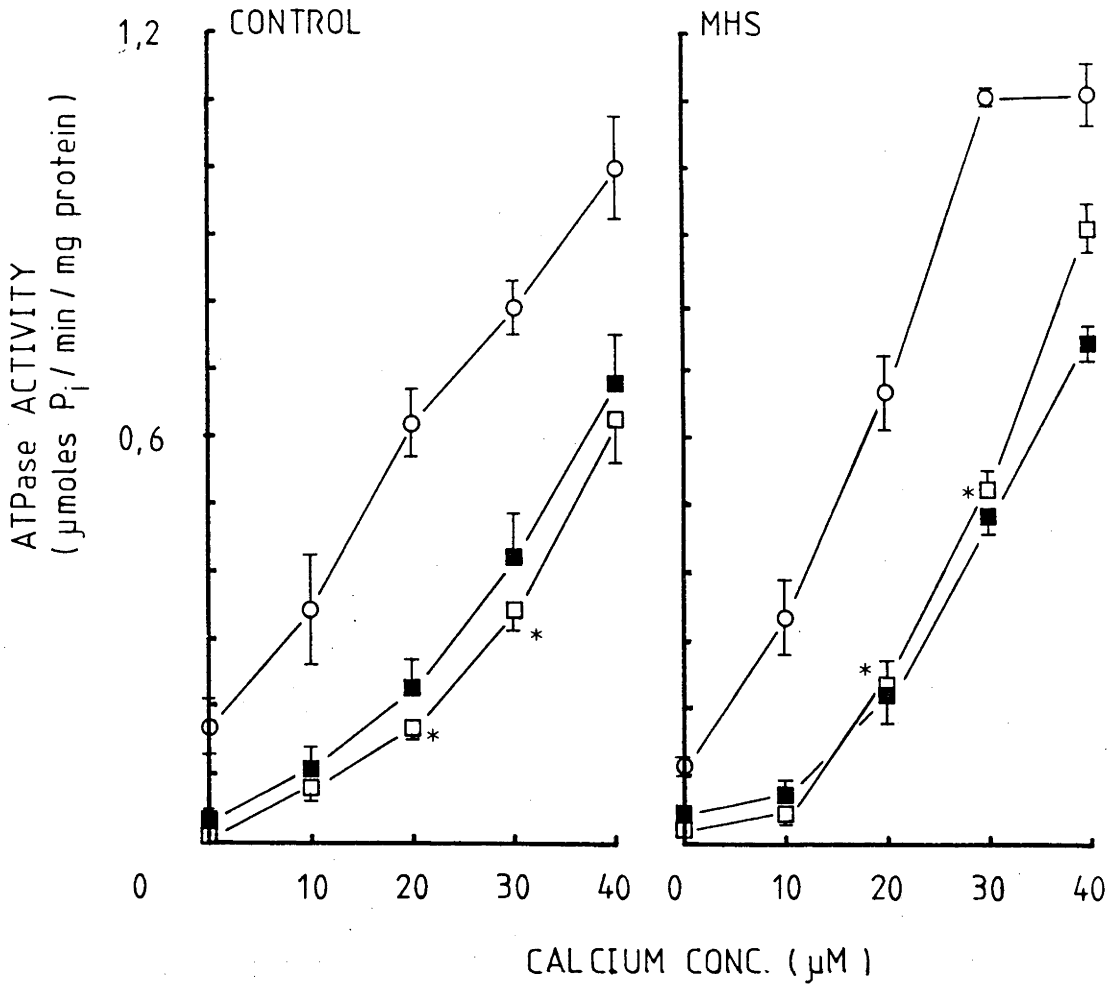


Figure 4.4 Effect of Dantrolene Sodium on the Inhibition of Ca^{2+}-Dependent ATPase Activity of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Trifluoperazine.

o No addition □ 150 µM TFP ■ 150 µM TFP + 20 µM dantrolene sodium

* 150 µM TFP value significantly less than the no addition value $p < 0.03$

Each value is the mean ± S.E. of 3 determinations. No 150 µM TFP + 20 µM dantrolene value is significantly different to the 150 µM TFP value.

Table 4.2 The Effect of an Ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetate wash on the Inhibition of Fragmented Sarcoplasmic Reticulum Ca^{2+}-dependent Adenosine Triphosphatase Activity Isolated from Control and Malignant Hyperpyrexia Susceptible Porcine Skeletal Muscle by Trifluoperazine

	ATPase Inhibition by TFP (% control)		
TFP concentration (μM)	100	200	500
Control FSR	30	55	99
Control EGTA-washed FSR	40	58	98
MHS FSR	31	55	97
MHS EGTA-washed FSR	37	56	97

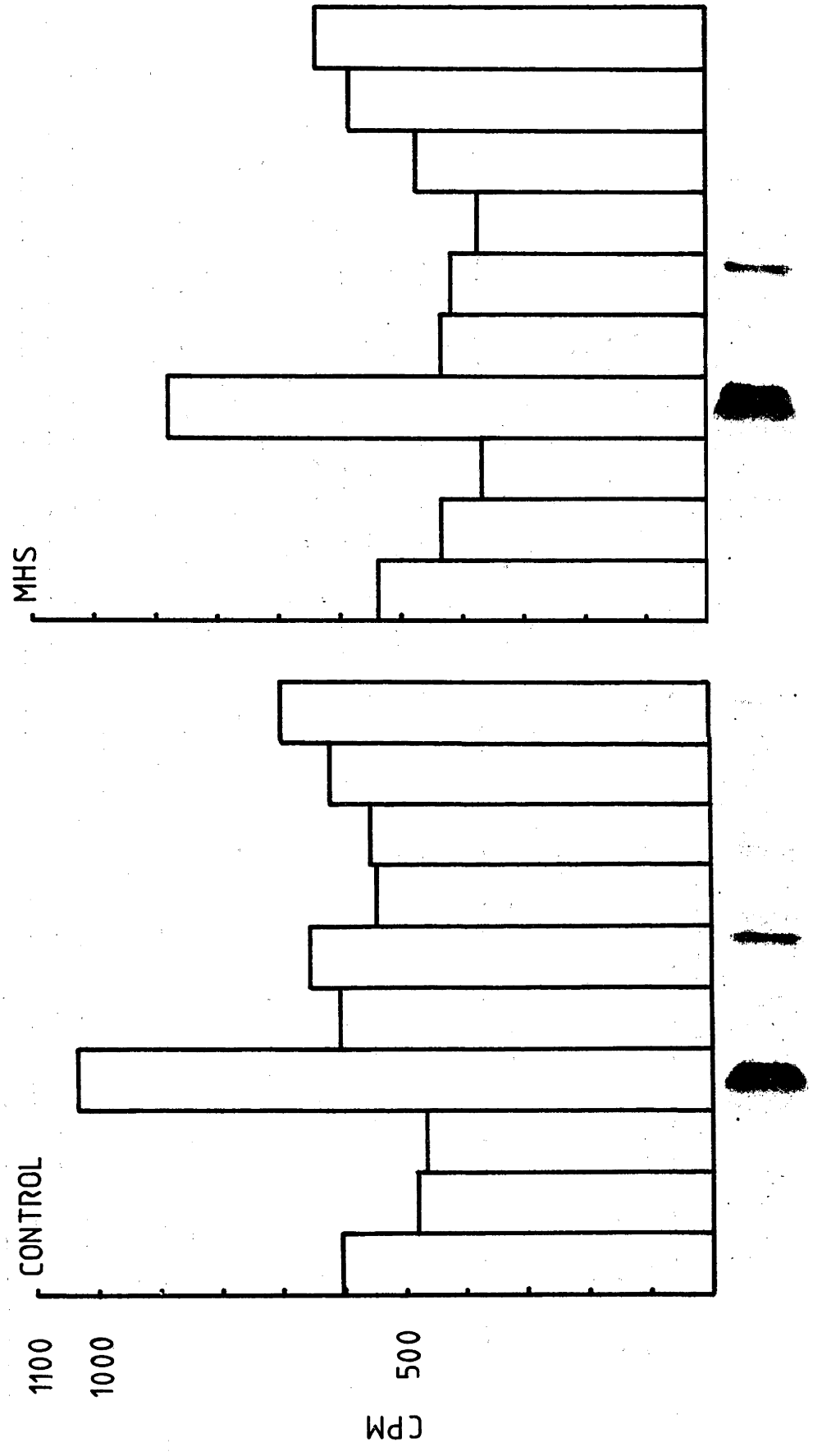
inhibition of control or MHS FSR Ca^{2+}-dependent ATPase activity by 150 μM TFP (Figure 4.4).

4.3.2.1 Ultra-violet Light-Activated Irreversible Binding of Tritiated Trifluoperazine to the Proteins of the Fragmented Sarcoplasmic Reticulum

The highly reactive free radical species of phenothiazine drugs is produced through oxidative metabolism, interaction with manganese or melanin, or by irradiation of the dissolved drug with UV light (Akera and Brody, 1972). This free radical form of CPZ has been shown to inhibit brain sodium and potassium activated ATPase by binding irreversibly to this protein (Akera and Brody, 1969, 1972). Prozialeck, Cimino and Weiss (1981) used UV light-activation of tritiated TFP to demonstrate a selective, Ca^{2+}-enhanced and irreversible binding of this labelled drug to calmodulin. Ultra-violet light-activated binding of tritiated TFP was used in the present study to investigate the protein specificity of interaction of TFP and FSR preparations, excluding calmodulin. The amount of calmodulin present in these preparations was too small to be identified using this technique.

In the present study FSR preparations isolated from control and MHS muscle were irradiated with UV light in the presence of Ca^{2+} and tritiated TFP. The FSR proteins were then separated using SDS-PAGE, the gels were sliced and the radioactivity associated with each slice was measured. No specificity of tritiated TFP interaction was observed in either control or MHS FSR preparations (Figure 4.5). The distributions of radioactivity along the gels correlated with the distribution of protein, the major protein component of the FSR being

Figure 4.5 Binding of UV Light-Activated Tritiated-Trifluoperazine to Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum Proteins.


MHS
CONTROL
1100
1000
500
CPM

the Ca^{2+}-dependent ATPase. In an attempt to saturate the Ca^{2+} -independent tritiated TFP free-radical binding sites of the FSR, control and MHS preparations were irradiated in the presense of unlabelled TFP and EGTA. Further irradiation in the presence of tritiated TFP and Ca^{2+} failed to demonstrate any Ca^{2+} -dependent specificity of binding, and the amount of radioactivity associated with the FSR proteins was reduced by half.

4.3.3 The Effects of R24571, Penfluridol, Pimozide, Fluphenazine, Chlorpromazine, Promethazine and Haloperidol on the Ca^{2+} -Dependent Adenosine Triphosphatase of Fragmented Sarcoplasmic Reticulum.

All the calmodulin antagonists tested in the present study (R24571, PEN, PIM, TFP, FPZ, CPZ, PRO and HPD) inhibited the FSR Ca^{2+} -dependent ATPase activity of control and MHS muscle (Table 4.3). Although individual drugs inhibited the Ca^{2+}-dependent ATPase activity of the FSR to different degrees, the extent of this inhibition was similar in both control and MHS preparations.

Penfluridol (Figure 4.6) and R24571 (Figure 4.7) were the most potent inhibitors of Ca^{2+}-dependent ATPase activity (I_{50} concentration approximately 20 μM). The I_{50} concentration for PIM was 80 μM (Figure 4.8), for TFP it was 250 μM (Figure 4.3), for FPZ it was approximately 220 μM (Figure 4.9), for CPZ it was approximately 385 μM (Figure 4.10) and for PRO and HPD it was greater than 500 μM (Figures 4.11 and 4.12, respectively).

Table 4.3 **Calmodulin Antagonist Drug Inhibition of Ca^{2+}-Dependent Adenosine Triphosphatase Activity from Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum.**

Drug	FSR ATPase I_{50}(μM) Control	FSR ATPase I_{50}(μM) MHS	Published Calmodulin I_{50}(μM)	Published Octanol/Water Partition Coefficient
R24571	26	18	0.35	-
Penfluridol	22	18	2.5	40,000,000
Pimozide	76	84	7	2,000,000
Trifluoperazine	250	250	10	1,700,000
Fluphenazine	275	165	10	912,000
Chlorpromazine	500	270	42	191,000
Haloperidol	500	500	60	555,000
Promethazine	500	500	340	22,400

(For references see Tables 1.2 and 2.1)

The effect of dantrolene sodium on the inhibition of Ca^{2+}-dependent ATPase activity of control and MHS FSR by HPD was investigated using the Ca^{2+}-dependent ATPase assay method b. (Materials and Methods 4.2.4, Results 4.3.2) Contractures induced in vitro by HPD were shown to be reversed by approximately 80% by dantrolene sodium in both control and MHS muscle (Chapter 2.3.3). The inhibition of the FSR Ca^{2+}-dependent ATPase activity of control and MHS preparations by HPD, however, did not appear to be affected by dantrolene sodium (Figure 4.13).

4.3.4 The Effects of Trifluoperazine, Haloperidol and Penfluridol on the Adenosine Triphosphate - Dependent Ca^{2+} Uptake by Fragmented Sarcoplasmic Reticulum.

The rates of ATP-dependent Ca^{2+} uptake by FSR preparations isolated from control and MHS porcine muscle did not differ significantly (Figure 4.14). The calmodulin antagonists TFP (Figures 4.15 and 4.16), HPD (Figures 4.17 and 4.18) and PEN (Figures 4.19 and 4.20) inhibited ATP-dependent Ca^{2+} uptake by the FSR. The extent of inhibition produced by these drugs was similar in both control and MHS preparations. Ca^{2+} uptake rates were inhibited by approximately 50% by 150 μM TFP, 150 μM PEN and 500 μM HPD.

Dantrolene sodium had no significant effect on the inhibition of ATP-dependent Ca^{2+} uptake by 150 μM TFP (Figures 4.15 and 4.16) or 500 μM HPD (Figures 4.17 and 4.18) in control or MHS FSR.

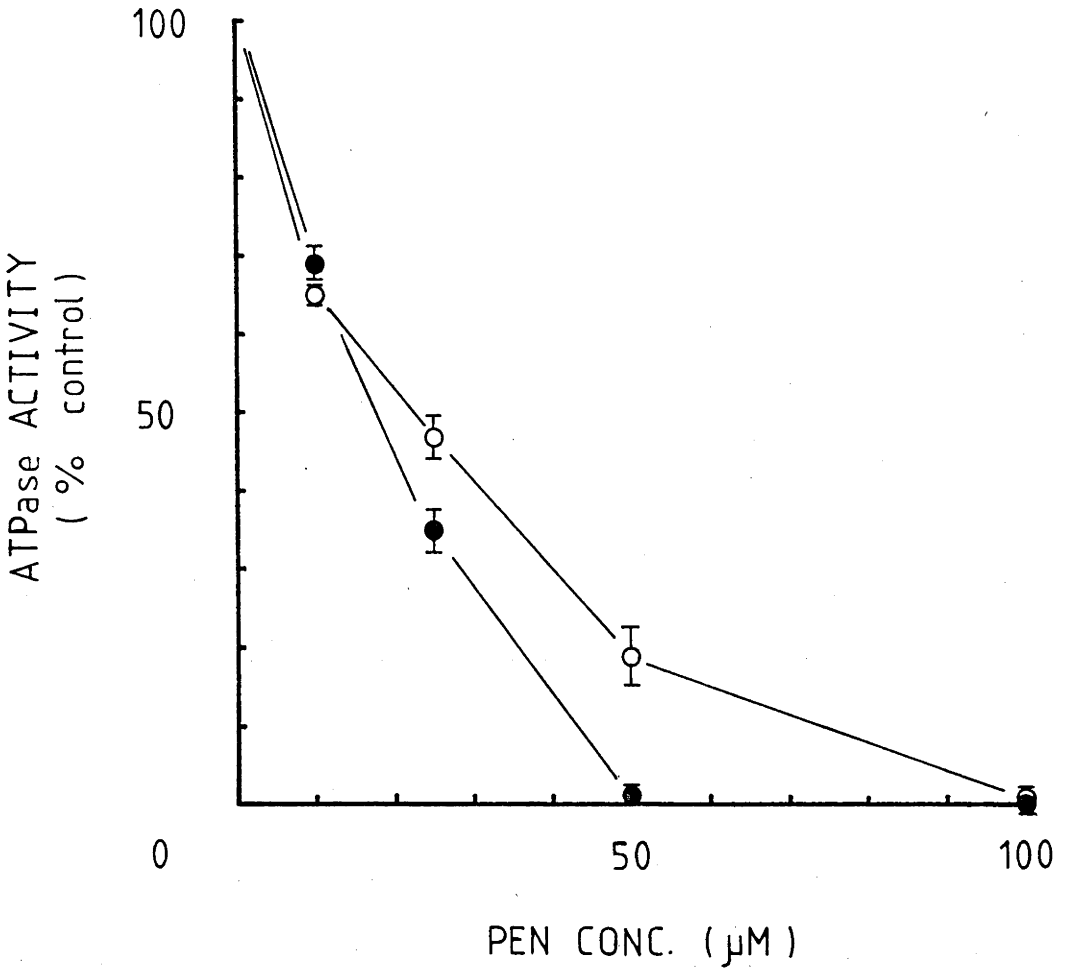


Figure 4.6 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Penfluridol.

o Control

• MHS

Each value is the mean ± S.E. of 3 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

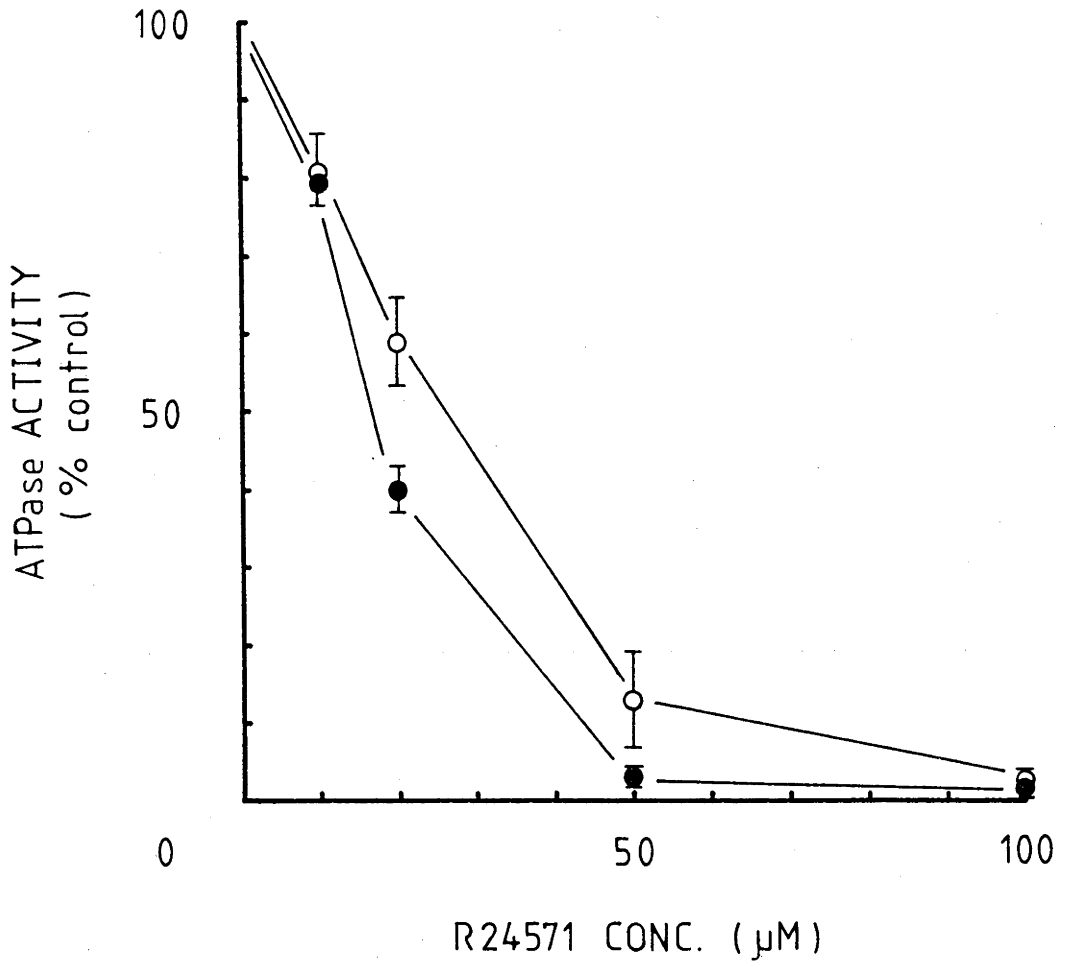


Figure 4.7 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by R24571.

o Control

• MHS

Each value is the mean ± S.E. of 3 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

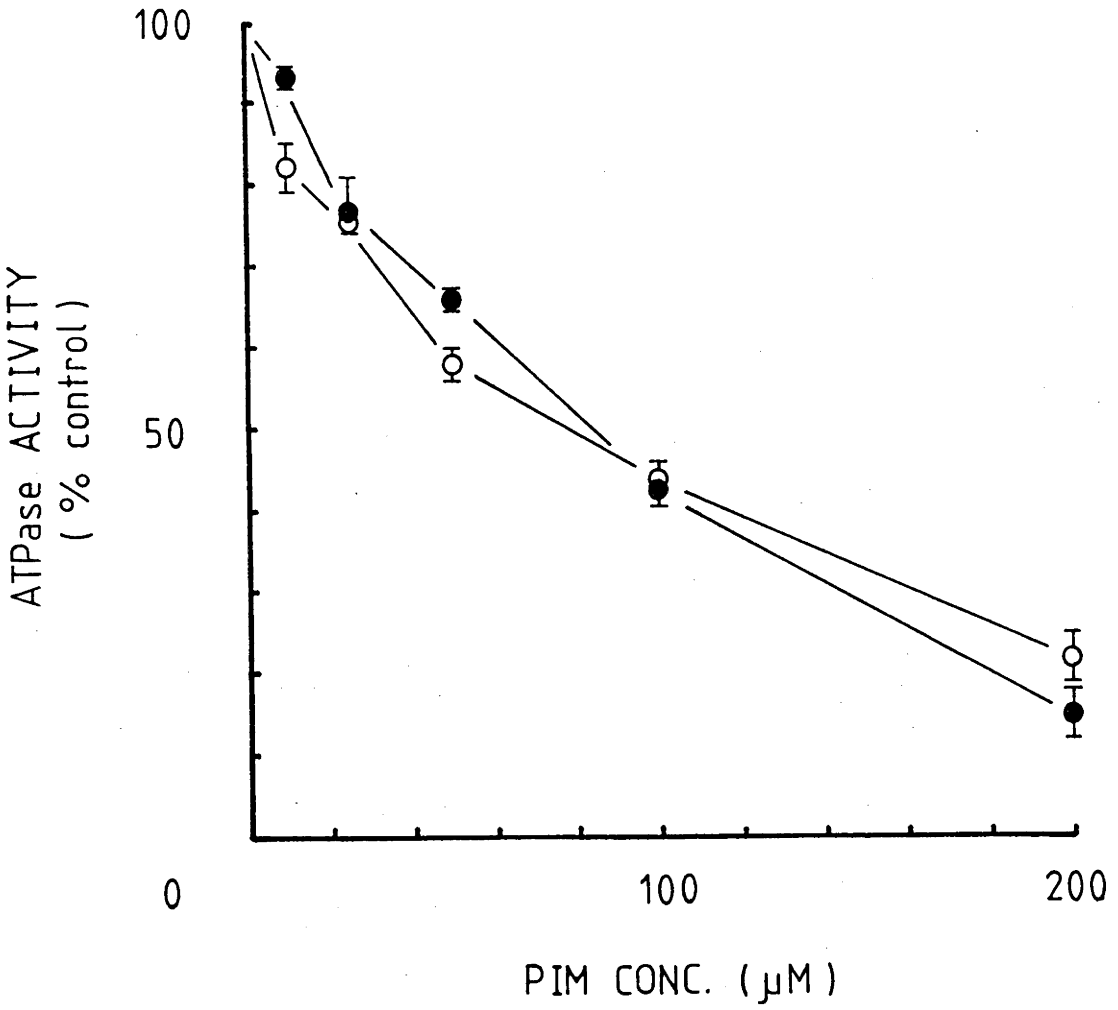


Figure 4.8 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Pimozide.

o Control

• MHS

Each value is the mean ± S.E. of 4 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

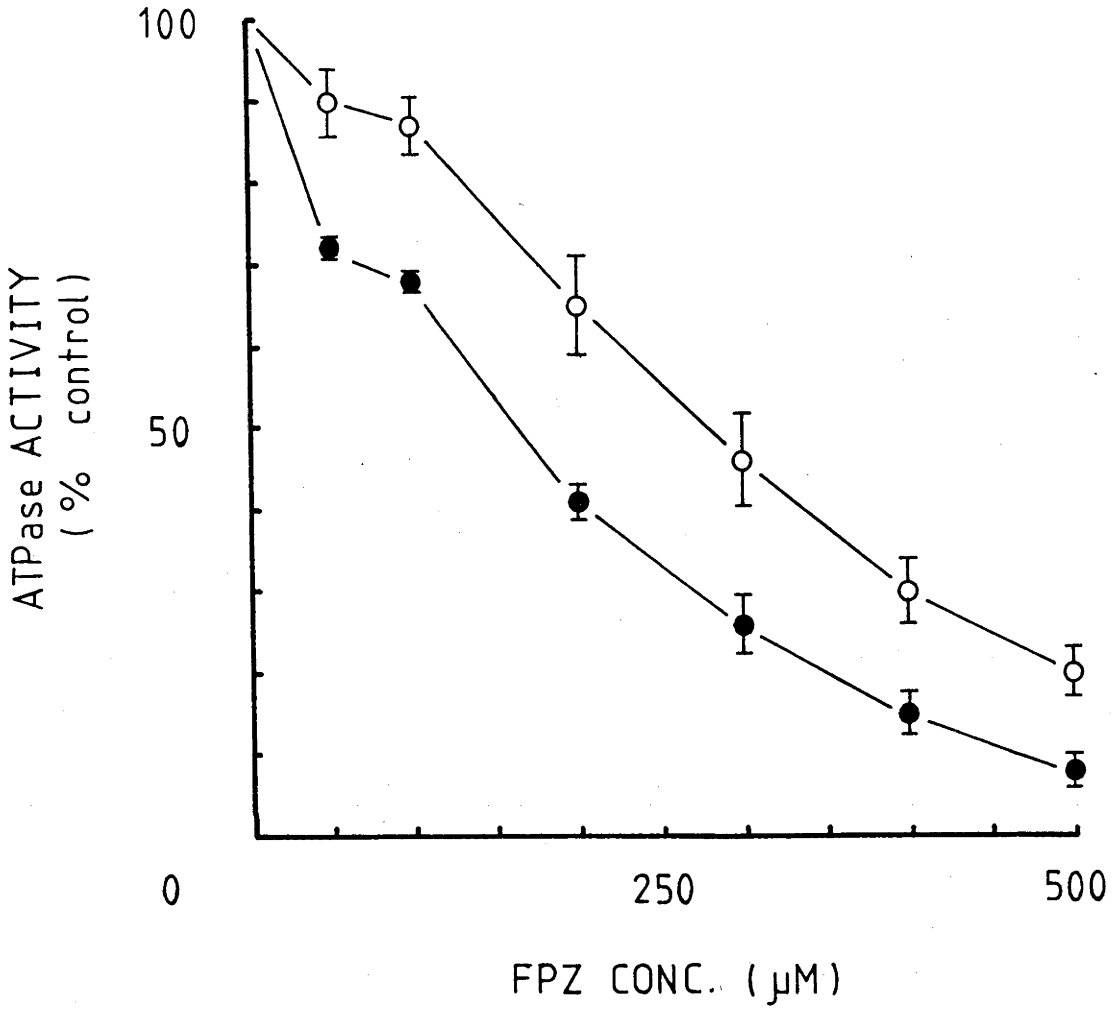


Figure 4.9 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Fluphenazine.

o Control

• MHS

Each value is the mean ± S.E. of 3 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

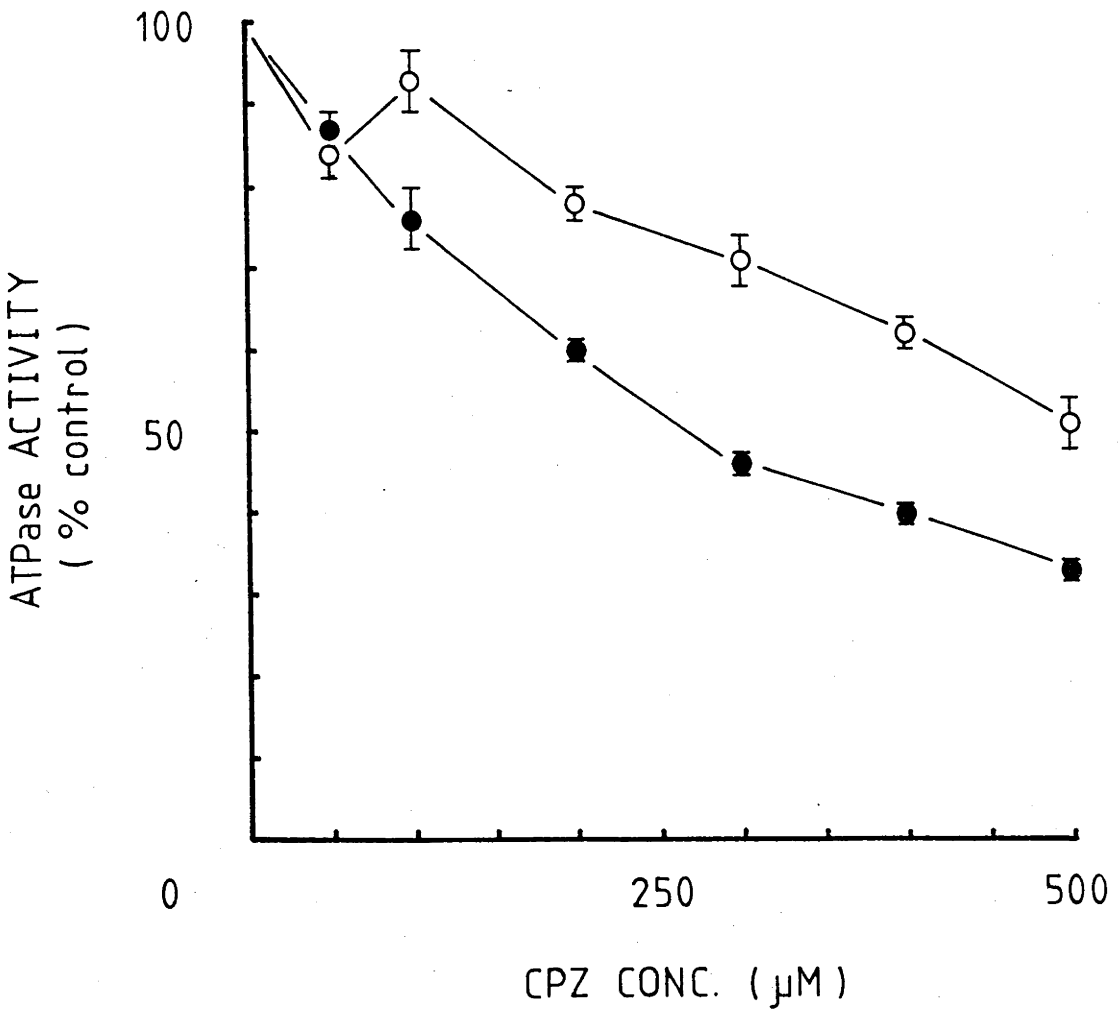


Figure 4.10 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Chlorpromazine.

o Control

• MHS

Each value is the mean ± S.E. of 3 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

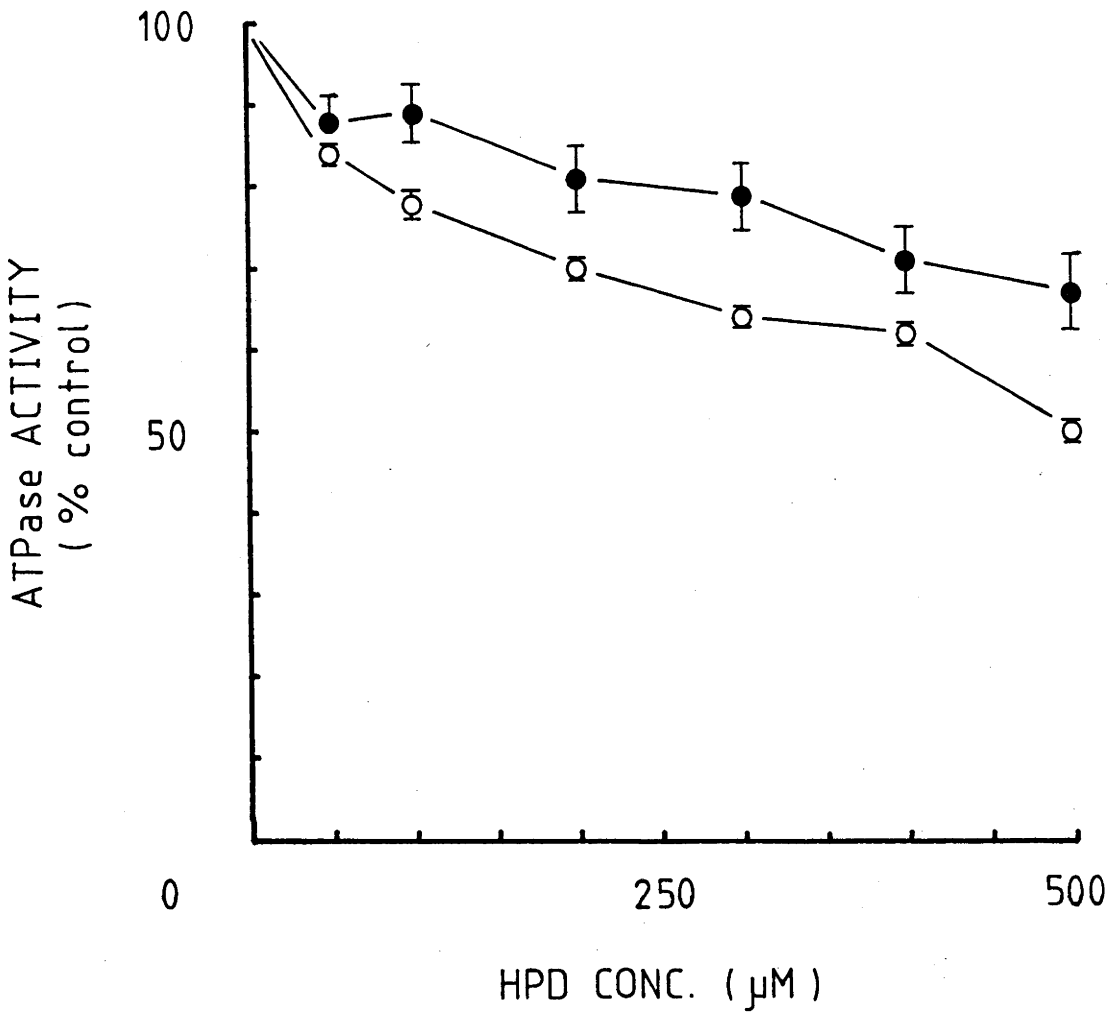


Figure 4.11 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Haloperidol.

o Control

• MHS

Each value is the mean ± S.E. of 3 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

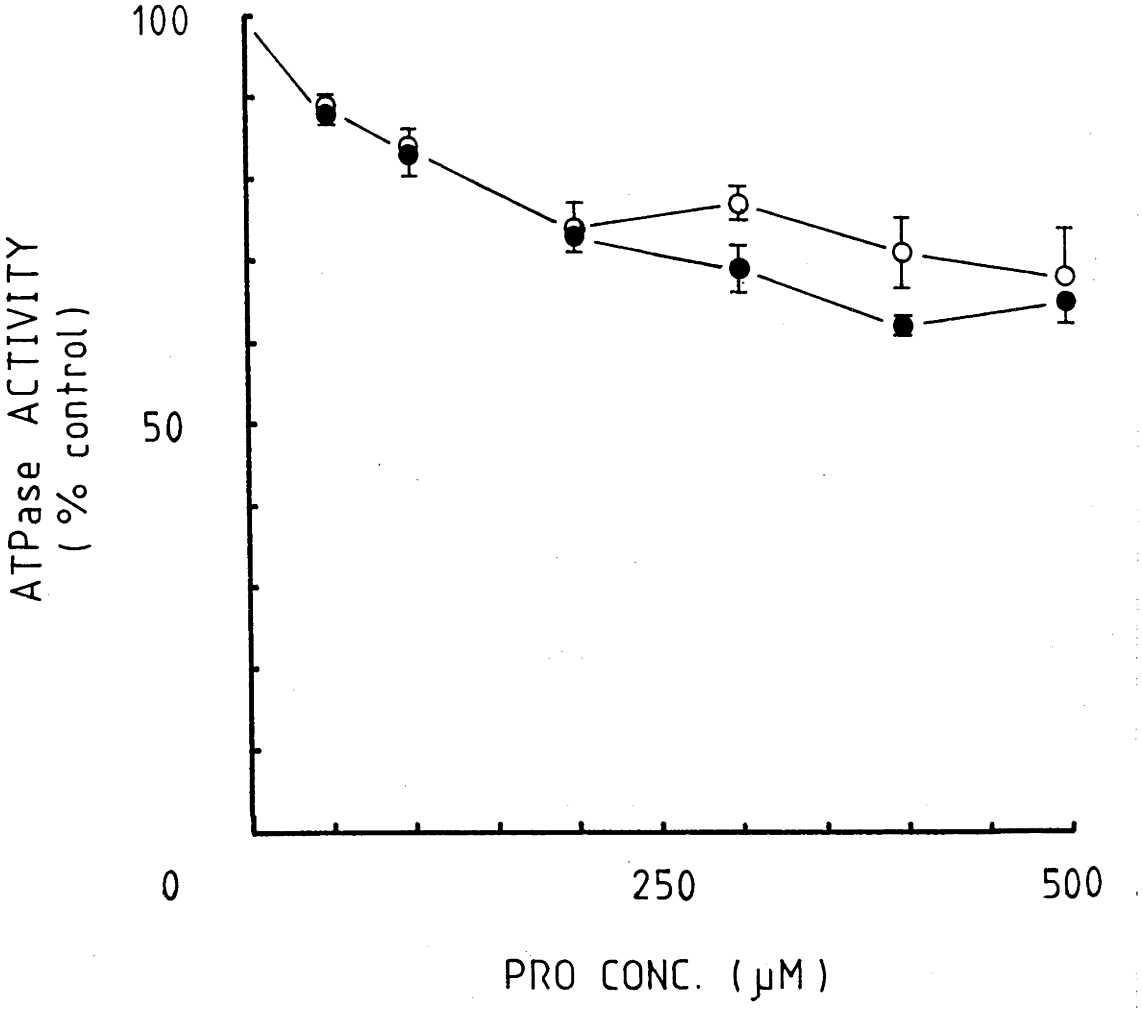


Figure 4.12 Inhibition of Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Promethazine.

o Control

• MHS

Each value is the mean ± S.E. of 3 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

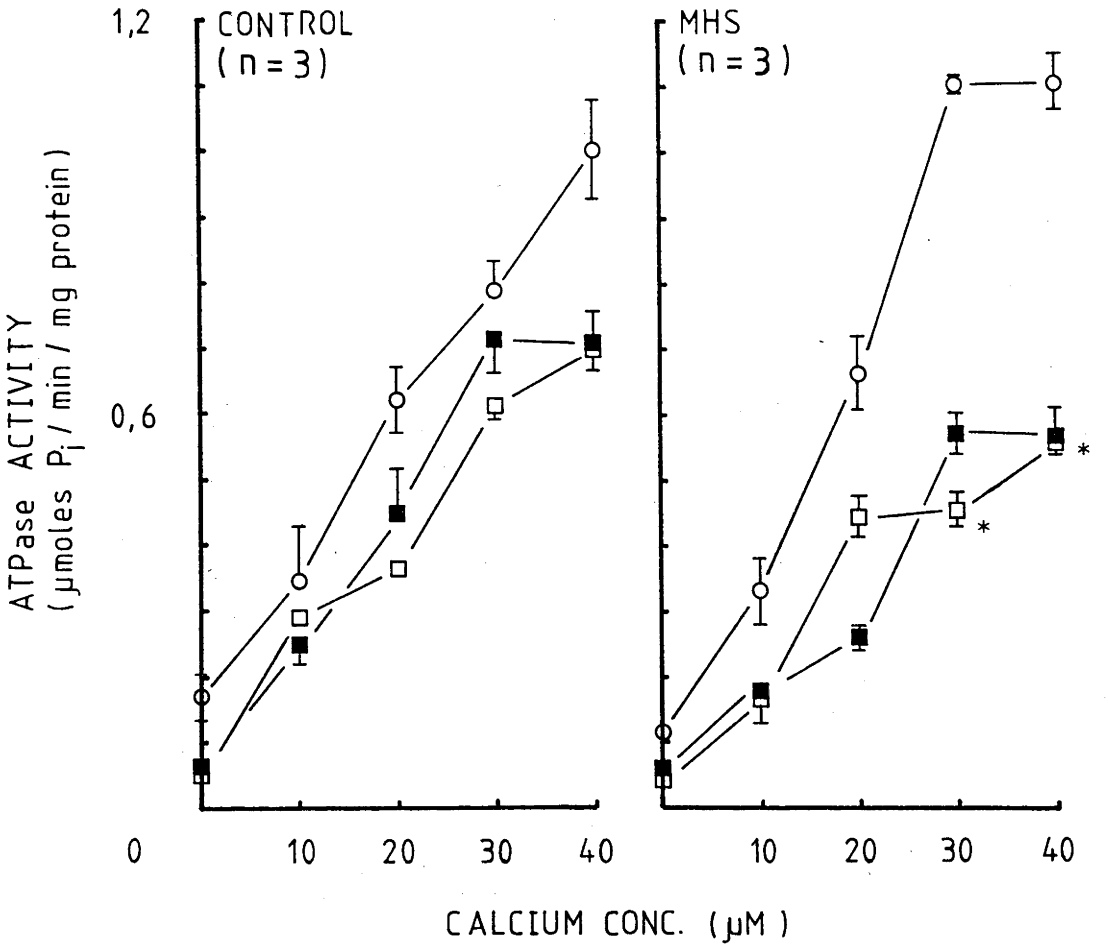


Figure 4.13 Effect of Dantrolene Sodium on the Inhibition of Ca^{2+}-Dependent ATPase Activity of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Haloperidol

o No addition □ 500 µM HPD ■ 500 µM HPD + 20 µM dantrolene sodium

* 500 µM HPD value is significantly less than no addition value $p < 0.01$

The differences between the results using 500 µM HPD and 500 µM HPD plus 20 µM dantrolene sodium are not statistically significant.

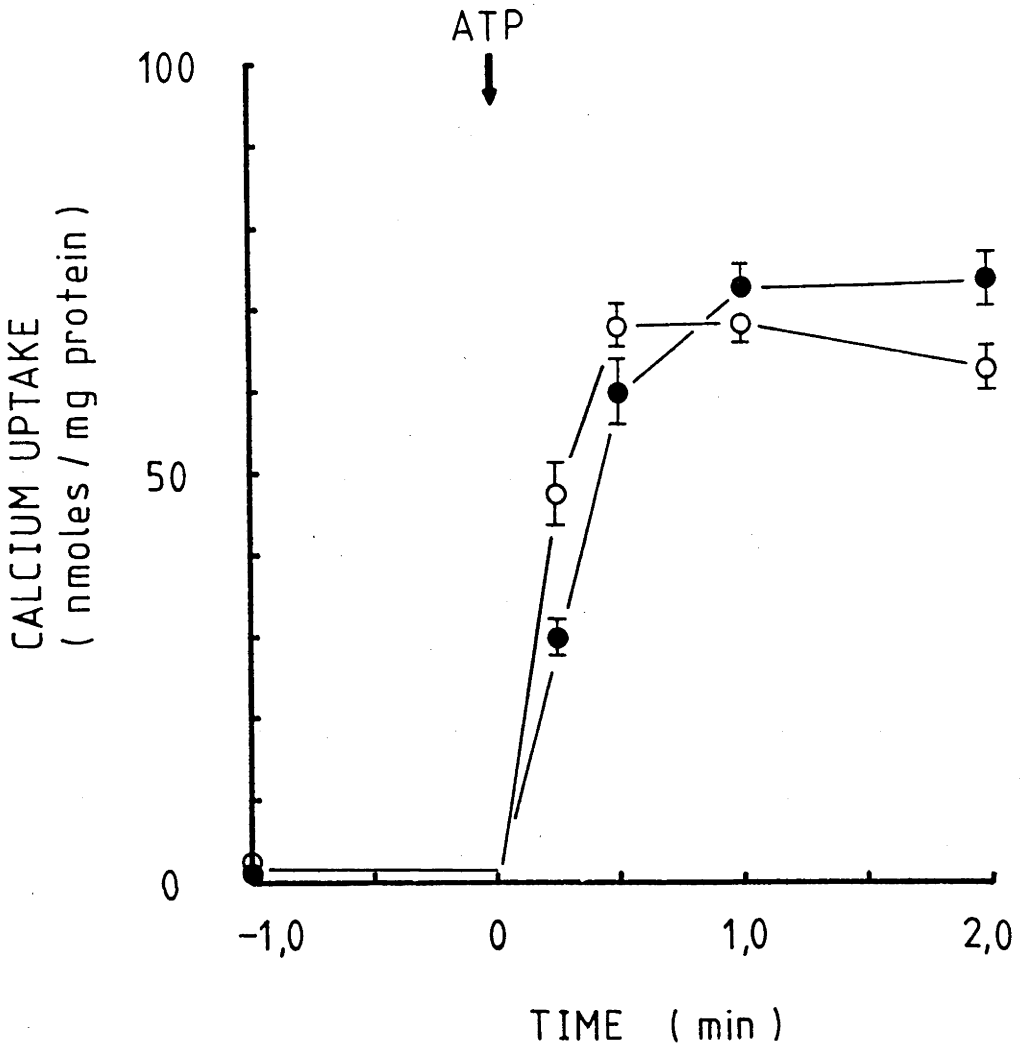


Figure 4.14 The ATP-Dependent Ca^{2+} Uptake by Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum.

o Control

• MHS

Each value is the mean ± S.E. of 9 determinations. The differences between the results in the control and MHS preparations of FSR are not statistically significant.

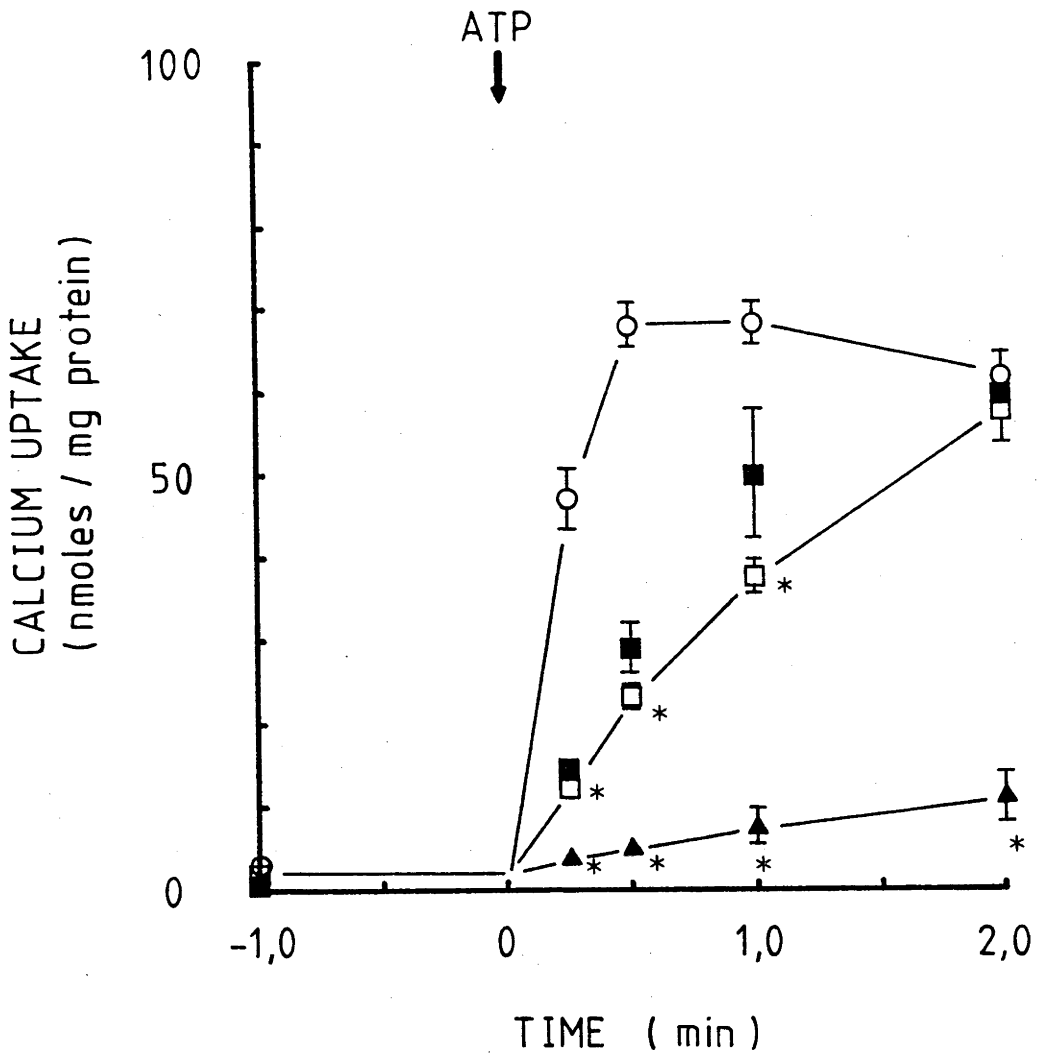


Figure 4.15 Inhibition of ATP-Dependent Ca^{2+} Uptake of Control Fragmented Sarcoplasmic Reticulum by Trifluoperazine.

o no addition (n=9) □ 150 μM TFP (n=10)

■ 150 μM TFP + 20 μM dantrolene sodium (n=3)

▲ 250 μM TFP (n=6)

Values shown are means ± S.E.

* 150 μM TFP value significantly less than no addition value $p < 0.001$

The differences between the results using 150 μM TFP and 150 μM TFP plus 20 μM dantrolene sodium are not statistically significant.

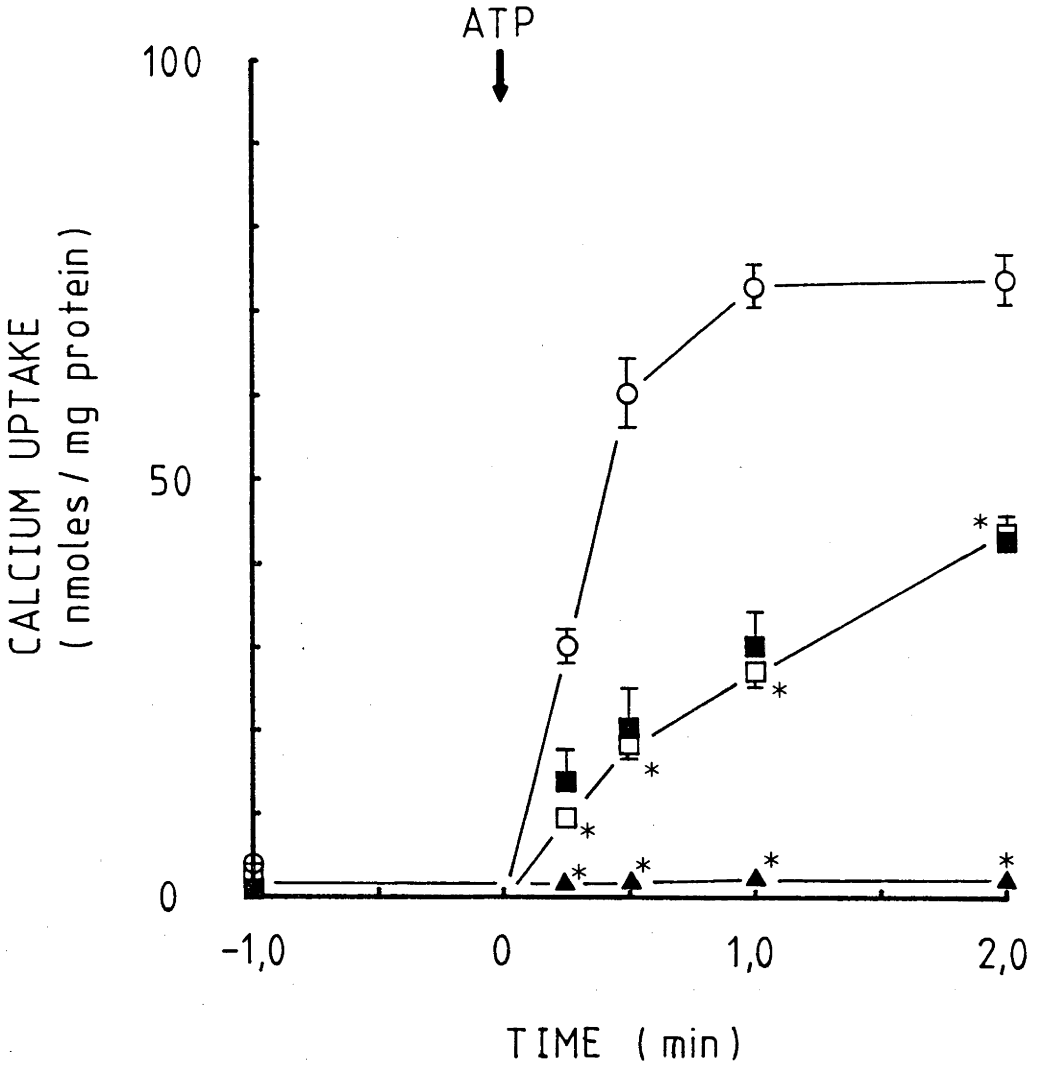


Figure 4.16 Inhibition of ATP-Dependent Ca^{2+} Uptake of Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Trifluoperazine.

o no addition (n=9) □ 150 μM TFP (n=9)

■ 150 μM TFP + 20 μM dantrolene sodium (n=3)

▲ 250 μM TFP (n=7)

Values shown are means ± S.E.

* 150 μM TFP value significantly less than no addition value $p < 0.002$

The differences between the results using 150 μM TFP and 150 μM TFP plus 20 μM dantrolene sodium are not statistically significant.

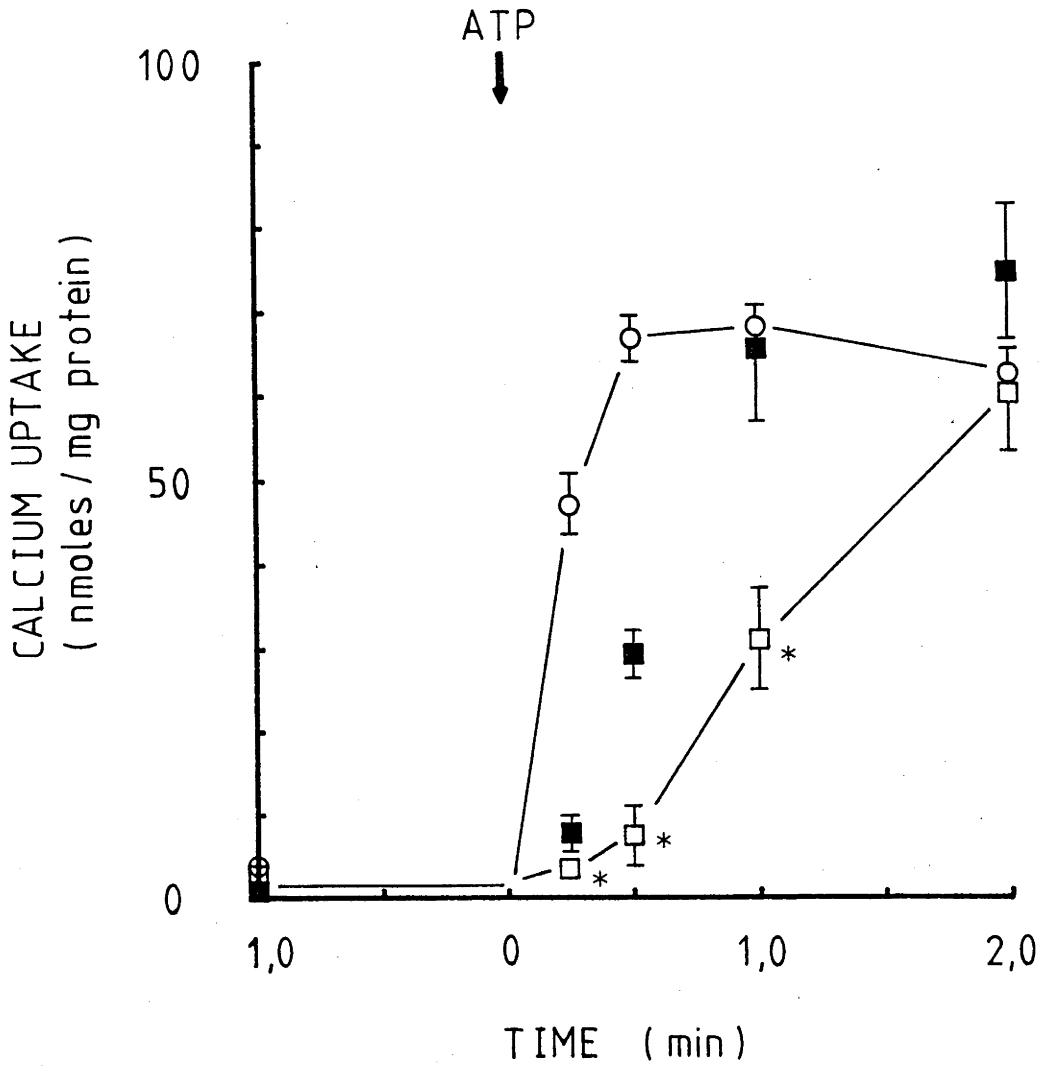


Figure 4.17 Inhibition of ATP-Dependent Ca^{2+} Uptake of Control Fragmented Sarcoplasmic Reticulum by Haloperidol.

o no addition (n=9) □ 500 μM HPD (n=3)

■ 500 μM HPD + 20 μM dantrolene sodium (n=3)

Values shown are means ± S.E.

* 500 μM HPD value significantly less than no addition value $p < 0.01$

The differences between the results using 500 μM HPD and 500 μM HPD plus 20 μM dantrolene sodium are not statistically significant.

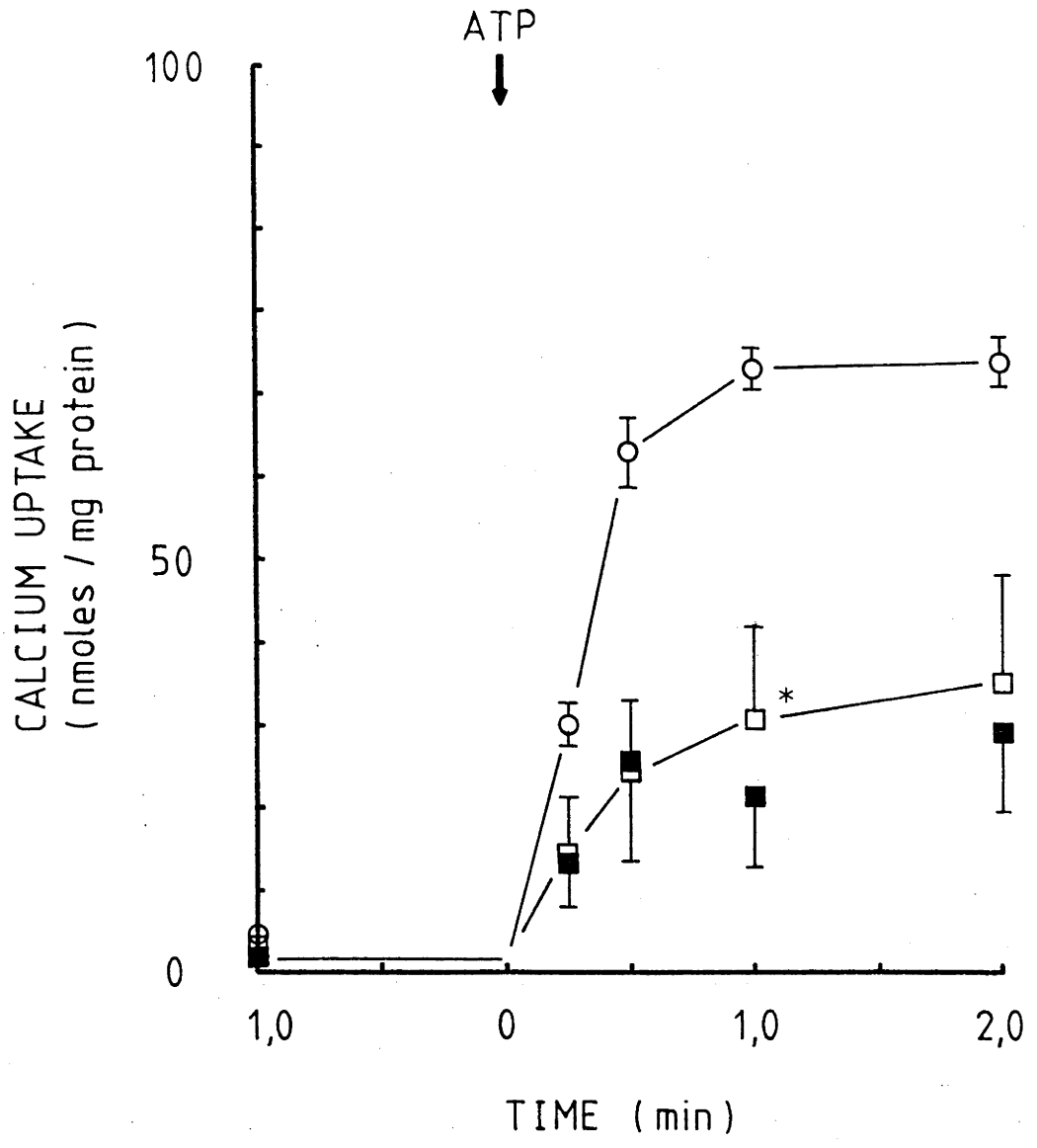


Figure 4.18 Inhibition of ATP-Dependent Ca^{2+} Uptake of Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Haloperidol.

o no addition (n=9) □ 500 μM HPD (n=3)

■ 500 μM HPD + 20 μM dantrolene sodium (n=3)

Values shown are means ± S.E.

* 500 μM HPD value significantly less than no addition value $p < 0.03$

The differences between the results using 500 μM HPD and 500 μM HPD plus 20 μM dantrolene sodium are not statistically significant.

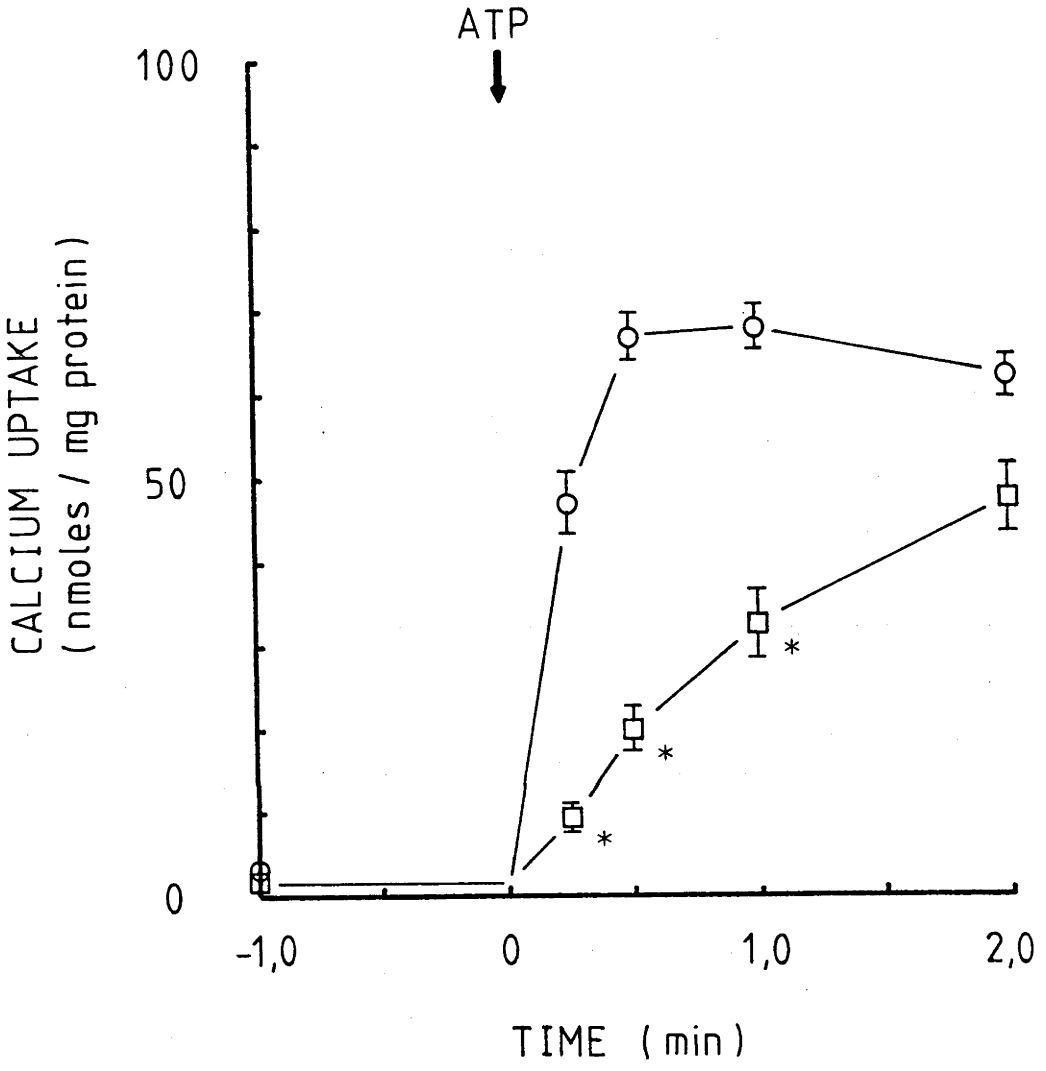


Figure 4.19 Inhibition of ATP-Dependent Ca^{2+} Uptake of Control Fragmented Sarcoplasmic Reticulum by Penfluridol.

o no addition (n=9) □ 150 μM PEN (n=3)

Values shown are means ± S.E.

* Significantly less than no addition value $p < 0.02$

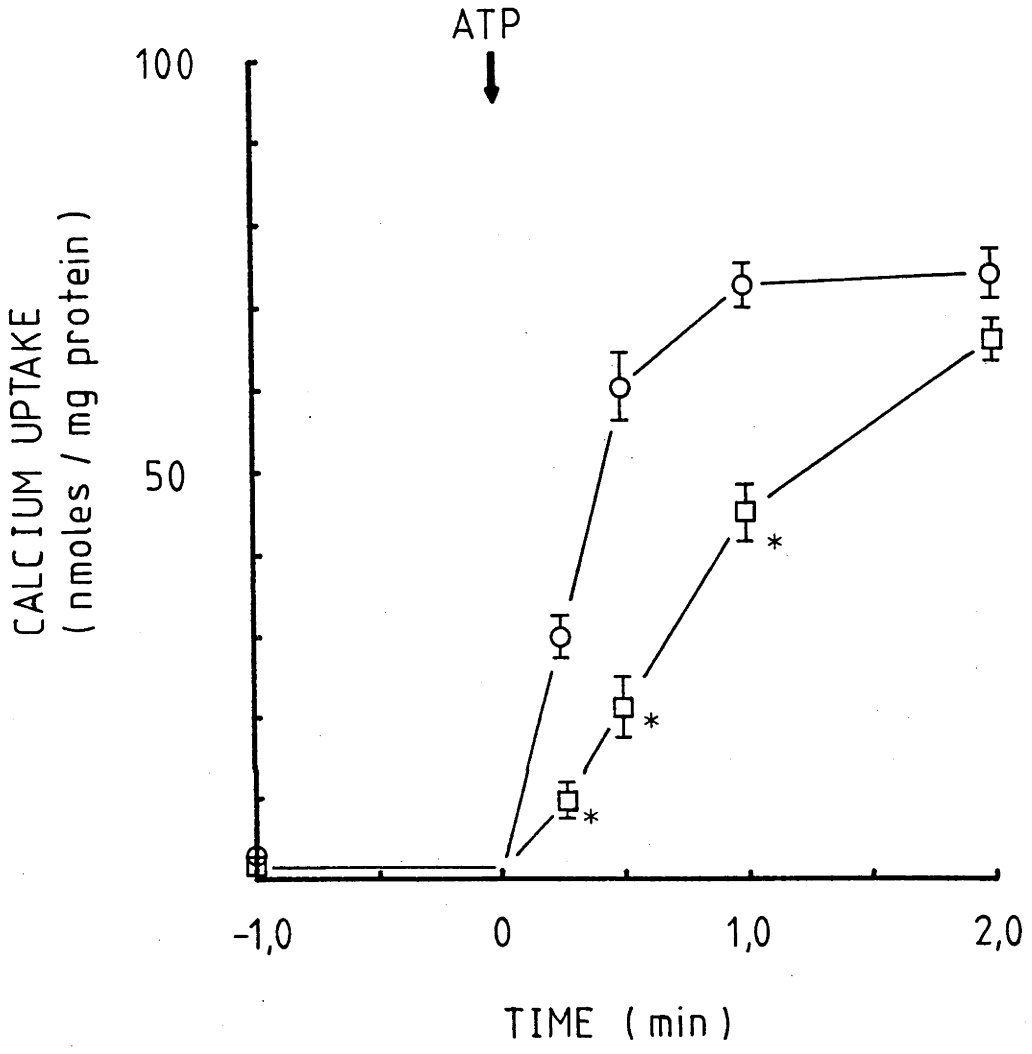


Figure 4.20 Inhibition of ATP-Dependent Ca^{2+} Uptake of Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Penfluridol.

o no addition (n=9) □ 150 μM PEN (n=3)

Values shown are means ± S.E.

* Significantly less than no addition value $p<0.04$

4.3.5 The Effects of Trifluoperazine and Haloperidol on the Equilibrium Efflux of Ca^{2+} from Fragmented Sarcoplasmic Reticulum.

The rates of efflux of Ca^{2+} from passively loaded FSR preparations isolated from control and MHS porcine muscle did not differ significantly (Figure 4.21). When 150 μM TFP (Figures 4.22 and 4.23) or 500 μM HPD (Figures 4.24 and 4.25) were added to the efflux experiments at time = 15 seconds the rates of Ca^{2+} efflux were significantly accelerated in both control and MHS preparations.

Dantrolene sodium had no significant effect on the acceleration of the efflux of Ca^{2+} caused by TFP (Figures 4.22 and 4.23) or HPD (Figures 4.24 and 4.25) from FSR preparations isolated from control or MHS muscle.

4.6.3 The Effect of Caffeine on the Ca^{2+}-Dependent Adenosine Triphosphatase Activity of Fragmented Sarcoplasmic Reticulum.

The effect of caffeine on the Ca^{2+}-dependent ATPase activities of control and MHS FSR was investigated using the Ca^{2+} -dependent ATPase assay method b (Materials and Methods 4.2.4, Results 4.3.2), since there have been reports that caffeine inhibited Ca^{2+}-dependent ATPase activities in skeletal muscle (Weber, 1968; Weber and Herz, 1968). In the present study, however, caffeine had no effect on control or MHS FSR Ca^{2+}-dependent ATPase activities (Figure 4.26).

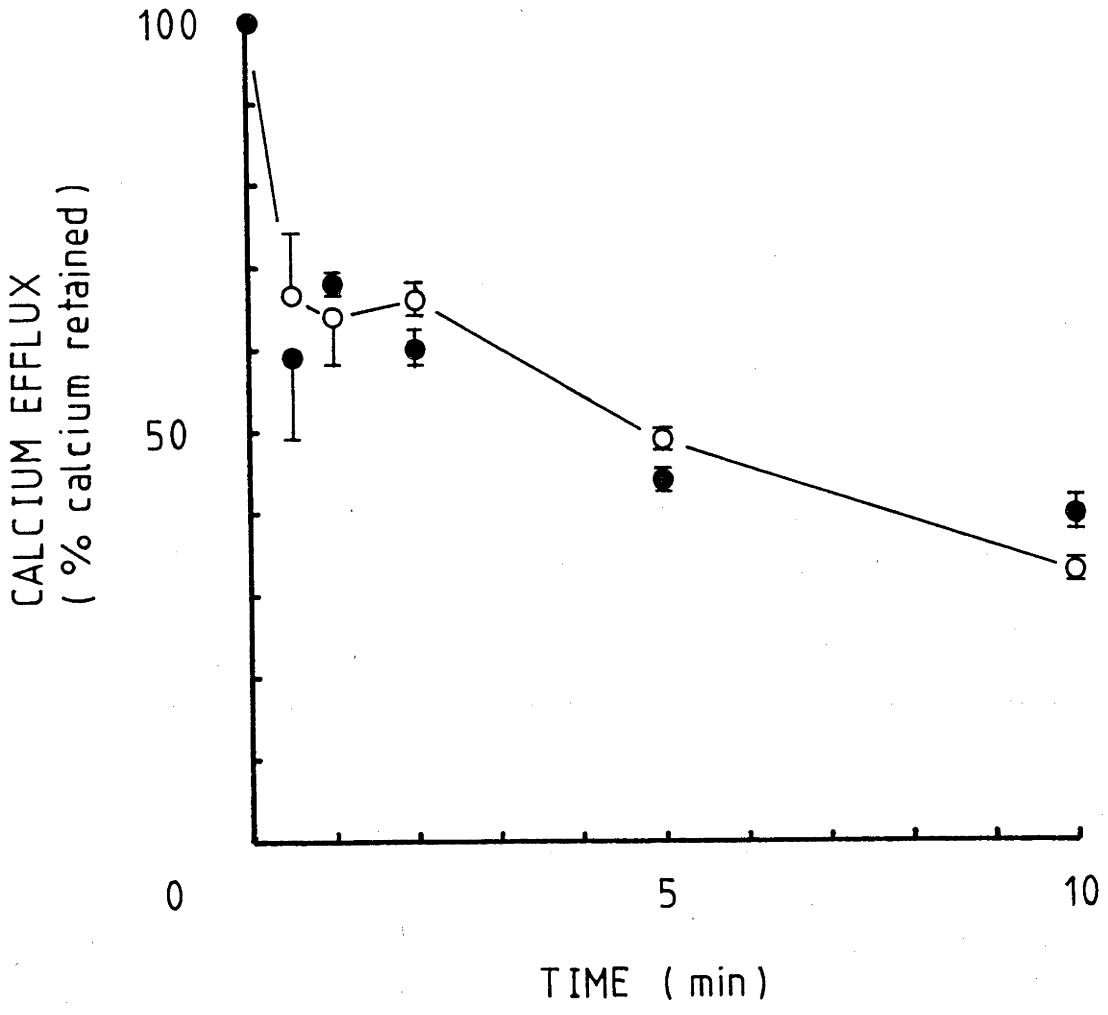


Figure 4.21 Calcium Efflux from Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum.

o Control (n=4)

• MHS (n=3)

Values shown are means ± S.E.

The differences between the results in the control and MHS preparations of FSR are not statistically significant.

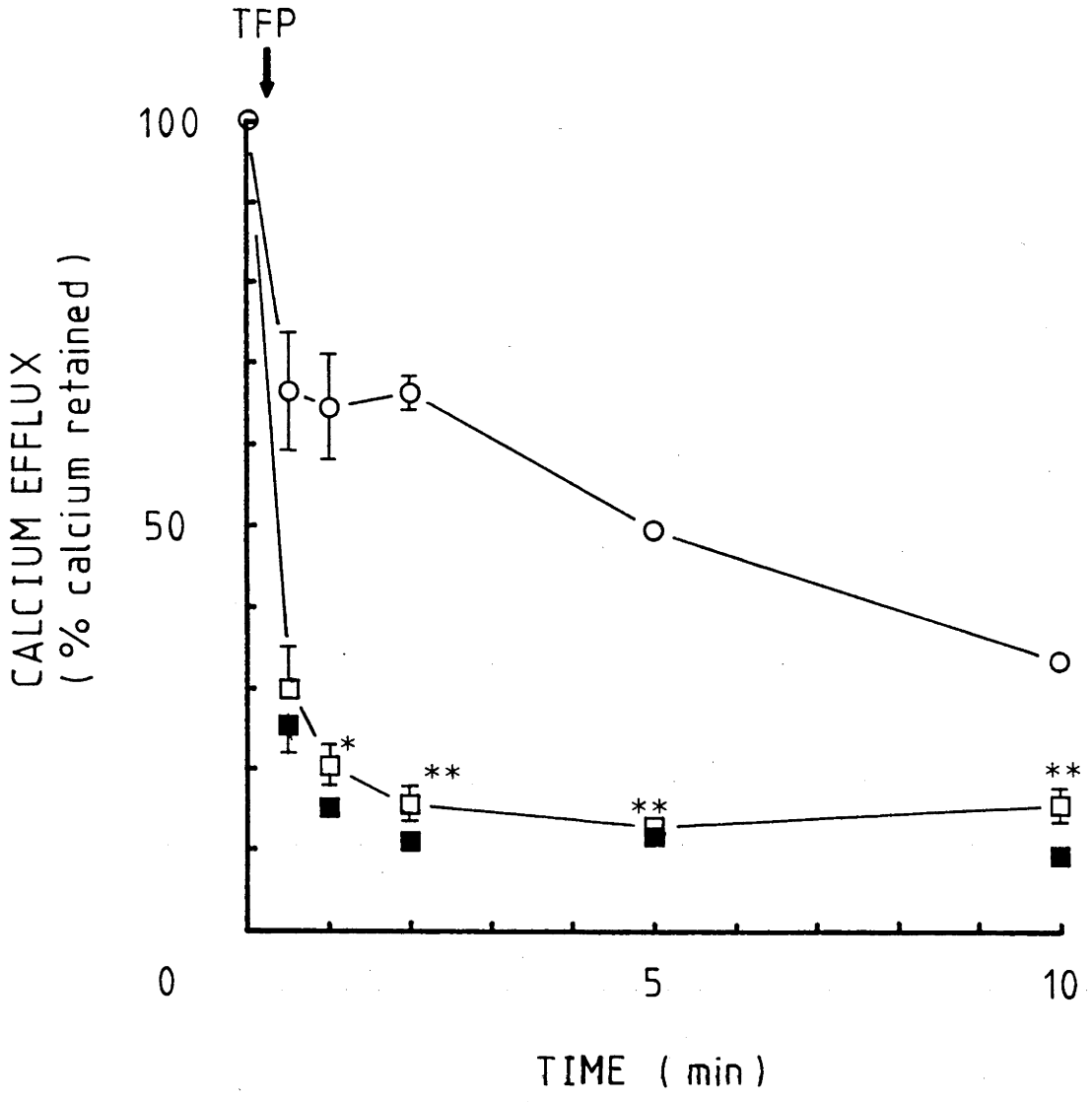


Figure 4.22 Acceleration of Ca^{2+} Efflux from Control Fragmented Sarcoplasmic Reticulum by Trifluoperazine.

o no addition (n=4) □ 150 μM TFP (n=3)

■ 150 μM TFP + 20 μM dantrolene sodium (n=3)

Values shown are means ± S.E.

* 150 μM TFP value significantly less than no addition value $p<0.04$

** 150 μM TFP value significantly less than no addition value $p<0.006$

The differences between the results using 150 μM TFP and 150 μM TFP plus 20 μM dantrolene sodium are not statistically significant.

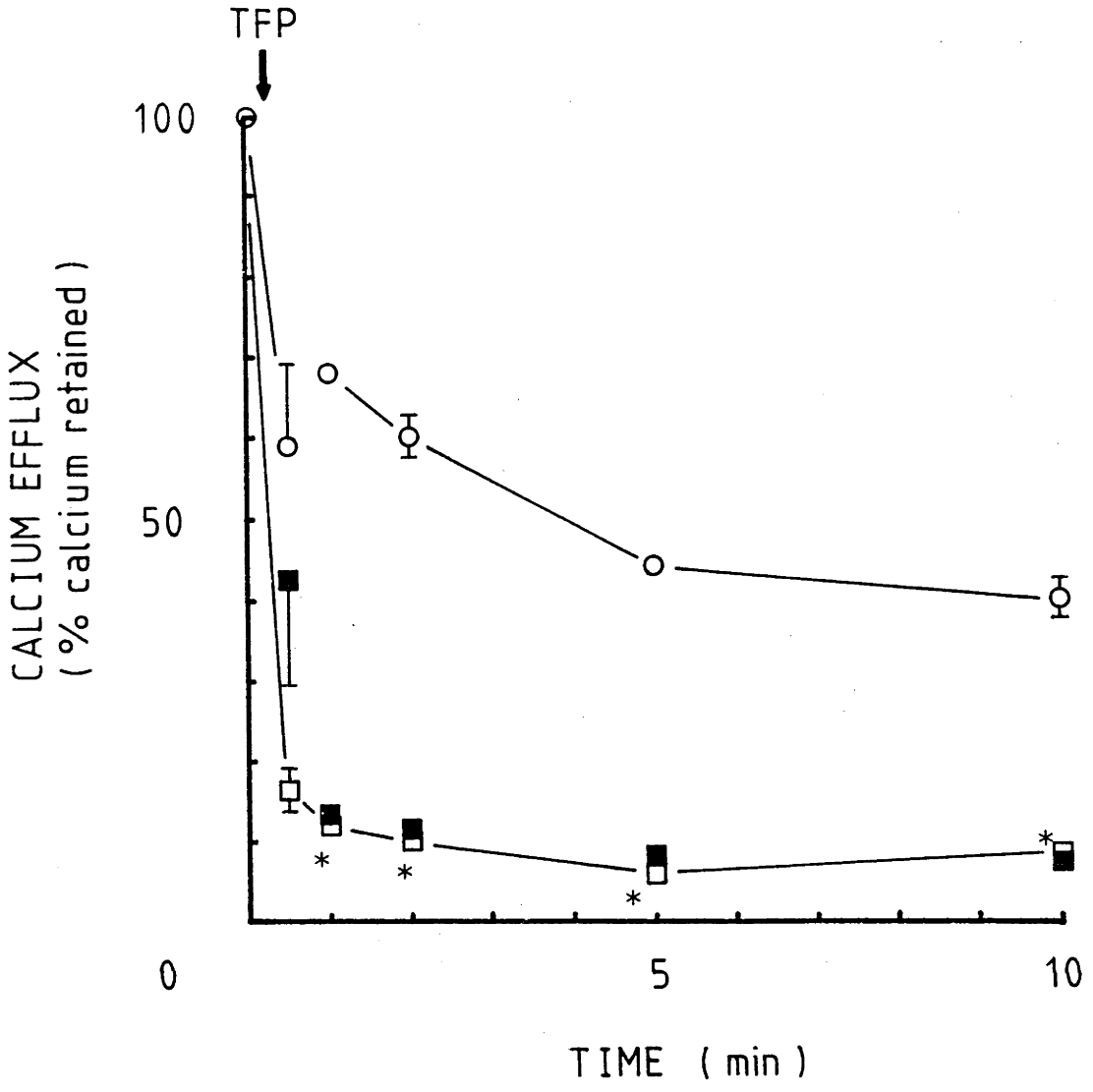


Figure 4.23 Acceleration of Ca^{2+} Efflux from Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Trifluoperazine.

o no addition (n=3) □ 150 μM TFP (n=3)

■ 150 μM TFP + 20 μM dantrolene sodium (n=3)

Values shown are means ± S.E.

* 150 μM TFP value significantly less than no addition value $p<0.005$

The differences between the results using 150 μM TFP and 150 μM TFP plus 20 μM dantrolene sodium are not statistically significant.

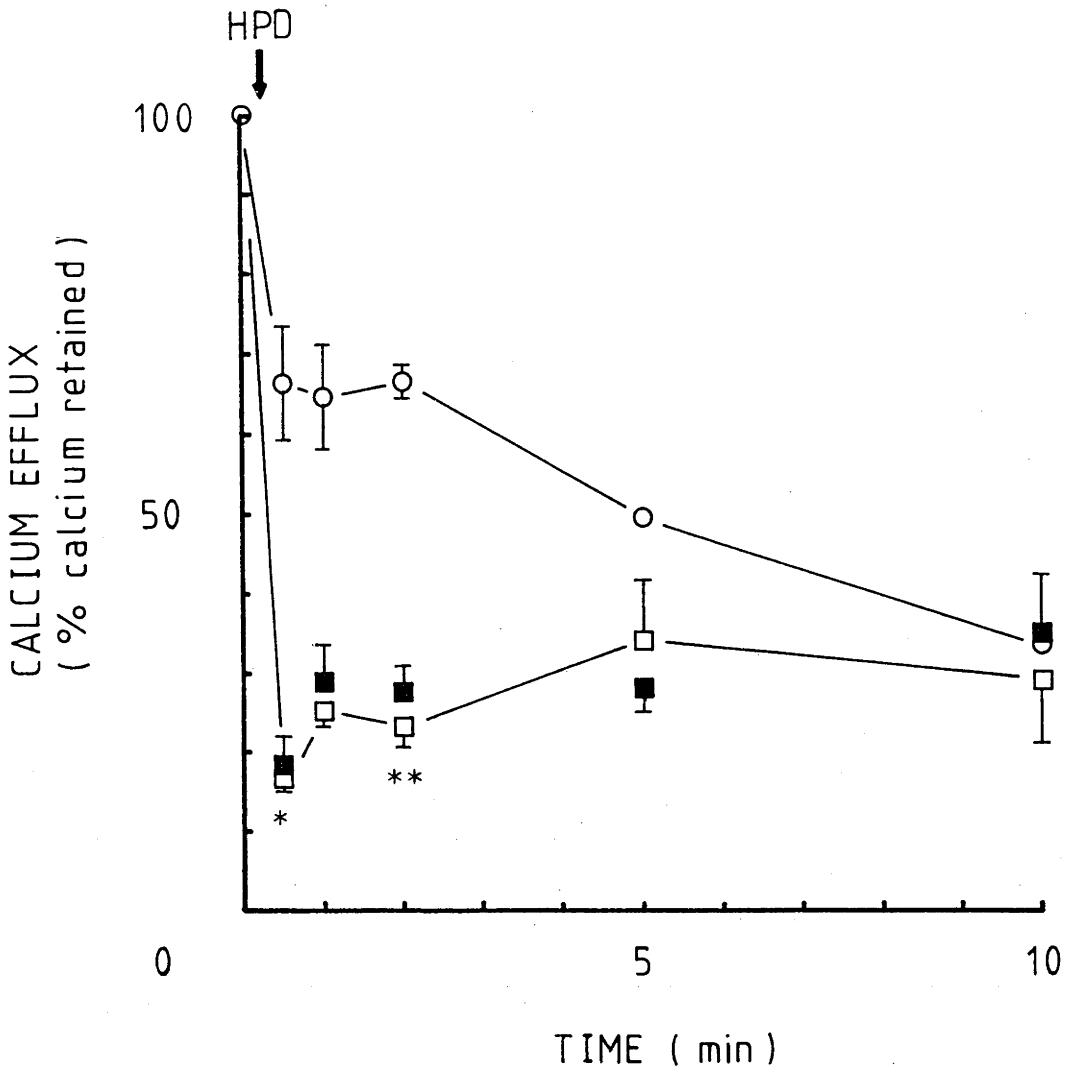


Figure 4.24 Acceleration of Ca^{2+} Efflux from Control Fragmented Sarcoplasmic Reticulum by Haloperidol.

o no addition (n=4) □ 500 μM HPD (n=3)

■ 500 μM HPD + 20 μM dantrolene sodium (n=3)

Values shown are means ± S.E.

* 500 μM HPD value significantly less than no addition value $p<0.04$

** 500 μM HPD value significantly less than no addition value $p<0.002$

The differences between the results using 500 μM HPD and 500 μM HPD plus 20 μM dantrolene sodium are not statistically significant.

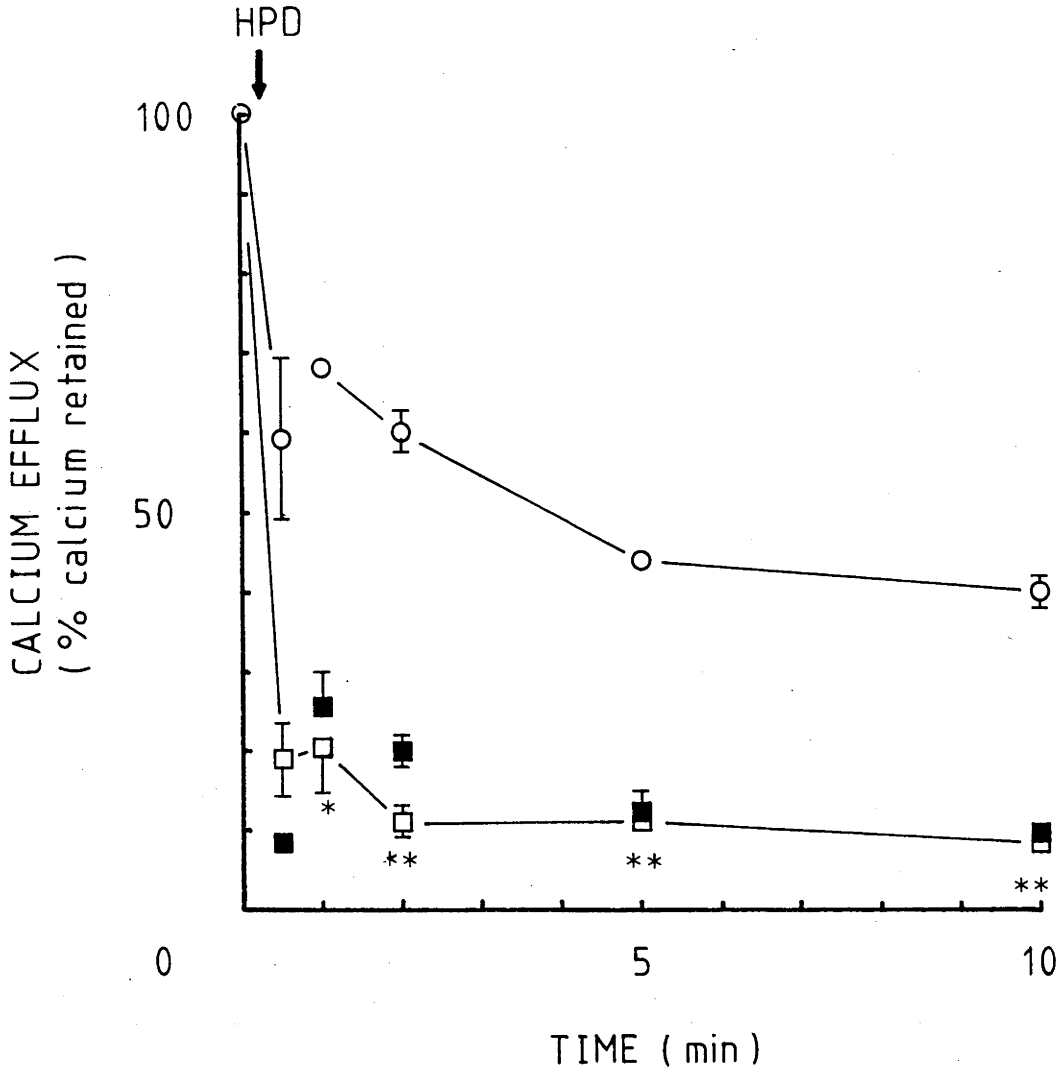


Figure 4.25 Acceleration of Ca^{2+} Efflux from Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum by Haloperidol.

o no addition (n=4) □ 500 μM HPD (n=3)

■ 500 μM HPD + 20 μM dantrolene sodium (n=3)

Values shown are means ± S.E.

* 500 μM HPD value significantly less than no addition value $p<0.02$

** 500 μM HPD value significantly less than no addition value $p<0.003$

The differences between the results using 500 μM HPD and 500 μM HPD plus 20 μM dantrolene sodium are not statistically significant.

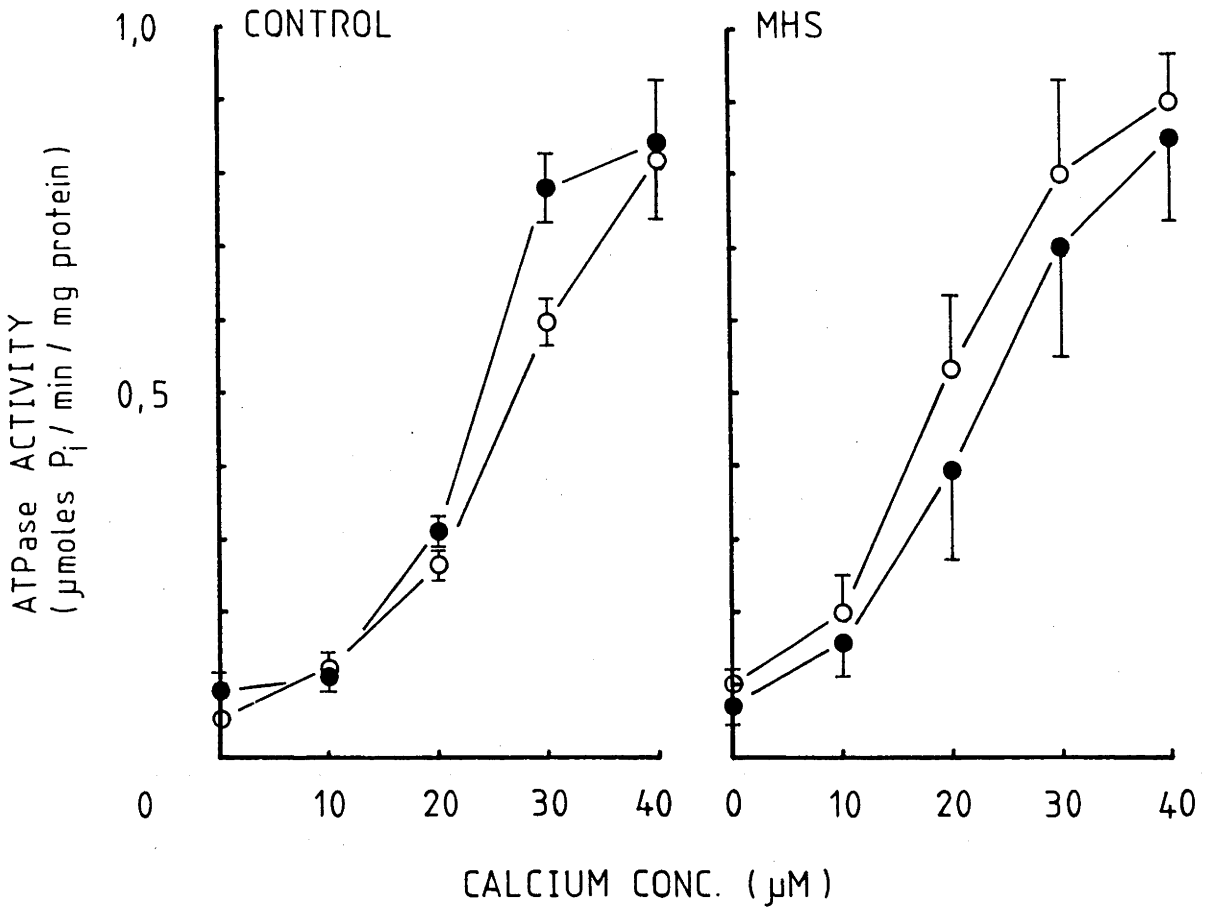


Figure 4.26 Effect of Caffeine on the Ca^{2+}-Dependent ATPase Activities of Control and Malignant Hyperpyrexia Susceptible Fragmented Sarcoplasmic Reticulum.

o no addition (n=3) ● 20 mM caffeine (n=3)

Values shown are means ± S.E.

The differences between the results in the control and control plus 20 mM caffeine values, and between the results in the MHS and MHS plus 20 mM caffeine values are not statistically significant.

4.4 Discussion

The results obtained in this chapter showed that there were no significant differences in calcium transport characteristics between FSR preparations isolated from control and MHS porcine skeletal muscle. The Ca^{2+}-dependent ATPase activities of control and MHS preparations were not significantly different, confirming earlier reports (McIntosh, Berman and Kench, 1977; Niebroj-Dobosz and Mayzner-Zawadzka, 1982; White, Collins and Denborough, 1983). The rates of ATP-dependent Ca^{2+} uptake did not differ significantly in control and MHS FSR preparations in agreement with three published reports (Nelson, Jones, Venable and Kerr, 1972; Denborough, Hird, King, Marginson, Mitchelson, Naylor, Rex, Zapf and Condron, 1973; White, Collins and Denborough, 1983). Other authors have observed MHS SR Ca^{2+} uptake to be reduced (McIntosh, Berman and Kench, 1977; Nelson, 1978; Gronert, Heffron and Taylor, 1979) or increased (Berman and Kench, 1973; Brucker, Williams, Popinigis, Galvez, Vail and Taylor, 1973; Britt, Endrenyi and Cadman, 1975) when compared to control SR preparations. These differences might be due to the various methods used to isolate the SR membranes and to quantitate Ca^{2+} uptake by these preparations. The equilibrium Ca^{2+} efflux characteristics of control and MHS FSR preparations were not significantly different, in agreement with an earlier published report (White, Collins and Denborough, 1983).

Caffeine causes contracture in skeletal muscle by releasing Ca^{2+} from the SR (Ogawa, 1970). It had also been reported that caffeine inhibited the SR Ca^{2+} uptake by inhibiting the SR Ca^{2+}-dependent ATPase activity of skeletal muscle (Weber, 1968; Weber and Herz,

1968). The present study, however, did not detect any inhibition of the Ca^{2+}-dependent ATPase activities of control or MHS FSR. Thus, an inhibition of the Ca^{2+}-dependent ATPase activity by caffeine was not involved in the induction of *in vitro* caffeine contractures in control or MHS porcine skeletal muscle. The difference in results between the earlier study (Weber, 1968) and the present investigation may have been due to the fact that Weber (1968) used frog skeletal muscle as the source of the SR membranes and carried out the ATPase assay at 24°C.

The Ca^{2+} transport characteristics of control and MHS FSR preparations did not differ in their responses to a range of drugs used in the present investigation as calmodulin antagonists. Calmodulin antagonist drugs inhibited the Ca^{2+}-dependent ATPase activity and ATP-dependent Ca^{2+} uptake of, and accelerated the Ca^{2+} efflux from, both control and MHS FSR to a similar extent. Trifluoperazine induced these effects on FSR Ca^{2+} transport at concentrations comparable to those reported for rabbit skeletal muscle SR (Campbell and MacLennan, 1982; Ho, Scales and Inesi, 1983).

The skeletal muscle relaxant dantrolene sodium did not reverse the effects of calmodulin antagonist drugs on control and MHS FSR Ca^{2+}-dependent ATPase activity, ATP-dependent Ca^{2+} uptake or Ca^{2+} efflux. This observation was consistent with a report that dantrolene sodium had no effect on these SR Ca^{2+} transport functions, in the absence of any drug, in control and MHS FSR preparations (White, Collins and Denborough, 1983).

The Ca^{2+}-dependent ATPase activity and ATP-dependent Ca^{2+} uptake of skeletal muscle SR have been shown to be calmodulin-independent (Chiesi and Carafoli, 1982). The mechanism of action by which the calmodulin antagonist drugs accelerated the efflux of Ca^{2+} from isolated FSR membranes may, however, have involved a specific interaction with calmodulin. Campbell and MacLennan (1982) and Chiesi and Carafoli (1983) have proposed that calmodulin may be involved in the release of Ca^{2+} from the SR. These proposals followed the observation of the calmodulin-dependent phosphorylation of a number of proteins associated with the SR membrane. It has been shown that these phosphoproteins did not interact directly with calmodulin, that the calmodulin-dependent kinase responsible for the phosphorylation of these proteins was membrane bound, and that at least one of these phosphoproteins was dephosphorylated by a calmodulin-dependent phosphatase activity (Chiesi and Carafoli, 1983). The exact function of the calmodulin-dependent kinase activity associated with the SR membrane has, however, yet to be determined.

All of the calmodulin antagonist drugs tested in the present investigation inhibited the Ca^{2+}-dependent ATPase activity of FSR preparations isolated from control and MHS porcine skeletal muscle, but with I_{50} concentrations much higher than those reported for the inhibition of a calmodulin-dependent function by these drugs (Tables 1.2 and 4.3). The inhibition of ATP-dependent Ca^{2+} uptake by, and the acceleration of Ca^{2+} efflux from, control and MHS FSR also required high concentrations of these drugs. These observations were consistent with the findings of Chiesi and Carafoli (1982) that the

Ca^{2+}-dependent ATPase and the ATP-dependent Ca^{2+} uptake of skeletal muscle SR were calmodulin-independent.

There are other possible mechanisms by which the calmodulin antagonist drugs might effect the Ca^{2+} transport characteristics of the SR membrane. One site of action proposed for TFP in skeletal muscle SR involved the 53,000 MW glycoprotein associated with isolated SR vesicles (Michalak, Campbell and MacLennan, 1980). This glycoprotein was present in a constant molar ratio with the Ca^{2+}-dependent ATPase enzyme of the SR and showed many similarities to the glycoprotein component of the sodium and potassium ATPase complex of the sarcolemma (Campbell and MacLennan, 1981). Chiesi and Carafoli (1982) proposed that the inhibition of the SR Ca^{2+}-dependent ATPase activity by TFP was correlated with the interaction of the drug with this glycoprotein, abolishing a glycoprotein mediated stimulation of ATPase activity. In the present investigation ultra-violet light-activated irreversible binding of tritiated TFP to the proteins of FSR preparations isolated from control and MHS muscle did not detect any specific TFP-protein interactions, however. The highly reactive TFP free radical appeared to bind to all the proteins present in these FSR preparations. This observation did not rule out a direct interaction between TFP and a protein or proteins to bring about its effects (for example, the 53,000 MW glycoprotein or the Ca^{2+}-dependent ATPase enzyme itself), but suggested that these interactions may be non-specific.

The relatively high concentrations of calmodulin antagonist drugs required to achieve substantial inhibition of the FSR Ca^{2+}-dependent ATPase and ATP-dependent Ca^{2+} uptake, and acceleration of Ca^{2+} efflux

from the FSR, suggested a non-specific effect of these drugs on this isolated membrane fraction. The well documented membrane effects of highly hydrophobic drugs such as the calmodulin antagonists may be sufficient to explain their effects on the SR Ca^{2+} transport functions (Guth and Spirtes, 1964; Seeman, 1972 and 1977; Frisk-Holmberg and Kleijn, 1972). The partitioning of large concentrations of calmodulin antagonist drugs into the FSR membranes seemed likely. Seeman (1977) observed that an aqueous phase concentration of CPZ of 10^{-5}M or 10^{-6}M established a membrane phase concentration of 20 mM. This amount of partitioning may effect SR membrane permeability or simply render it leaky to Ca^{2+}. Ho, Scales and Inesi (1983) concluded that the inhibition by TFP of the SR Ca^{2+}-dependent ATPase activity and ATP-dependent Ca^{2+} uptake of rabbit skeletal muscle were due to structural perterbations induced by the drug partitioning into the SR membrane. These authors have demonstrated ultrastructural alterations including SR membrane thickening and loss of granular detail on the outer surface of SR vesicles in the presence of TFP. Also, because of the highly hydrophobic nature of the calmodulin antagonist drugs, they may interact directly with the hydrophobic regions of membrane proteins such as the SR Ca^{2+}-dependent ATPase enzyme.

4.5 Summary

The calmodulin antagonist drugs affected the contractile characteristics of both control and MHS skeletal muscle *in vitro* at concentrations which might produce disruption of the Ca^{2+} transport functions of the SR. These effects were studied using fragmented SR preparations isolated from control and MHS porcine skeletal muscle.

The FSR Ca^{2+}-dependent ATPase activities and ATP-dependent Ca^{2+} uptake rates and the rates of efflux of Ca^{2+} from FSR isolated from control and MHS porcine skeletal muscle did not differ significantly. A range of calmodulin antagonist drugs inhibited the FSR Ca^{2+}-dependent ATPase activity and ATP-dependent Ca^{2+} uptake, and accelerated the efflux of Ca^{2+} from FSR preparations isolated from control and MHS muscle. The extent to which the calmodulin antagonist drugs affected these FSR Ca^{2+} transport functions was similar in both control and MHS preparations.

The dissruption of the Ca^{2+} transport functions of both control and MHS FSR by calmodulin antagonist drugs was probably due to a combination of the following mechanisms:

a) specific interaction with a calmodulin-dependent function such as the calmodulin-dependent protein kinase system associated with the SR

b) other specific interactions such as binding to the 53,000 MW glycoprotein associated with the Ca^{2+}-dependent ATPase enzyme

c) non-specific interactions with SR membrane lipids and proteins due to the highly hydrophobic nature of these drugs.

CHAPTER 5 THE NEUROLEPTIC MALIGNANT SYNDROME AND MALIGNANT HYPERPYREXIA.

5.1 Introduction

The Neuroleptic Malignant Syndrome (NMS) was first recognized as a serious side effect of neuroleptic therapy in psychiatric patients in 1960 (Delay, Pichot and Lemperiere, 1960). The symptoms of NMS are similar to those of MH (Table 5.1) and include hyperpyrexia, hypertonicity, tachycardia, an elevated serum CPK and labile blood pressure (Caroff, 1980). Although the incidence of NMS was not known, the number of cases found in a series of several hundred psychiatric patients suggested an incidence of 0.5-1% (Delay, Pichot and Lemperiere, 1963). The mortality rate for NMS has been estimated at 30% (Burke, Fahn, Mayeux, Weinberg, Louis and Willner, 1981).

The neuroleptic drugs which have been implicated in causing NMS episodes are of the high potency class and include haloperidol (Weinberg and Kelly, 1977; Feibel and Schiffer, 1981), phenothiazines such as fluphenazine (Allan and White, 1972), chlorpromazine (Morris, McCormick and Reinarz, 1980) and trifluoperazine (Smego and Durack, 1982), thiothixene (Weinberg and Twersky, 1983), and combinations of the above drugs (Oppenheim, 1973). Phenothiazines in conjunction with morphine (Bleichner, Squara and Parent, 1981) and alcohol (Freed, 1981) have also been implicated in the genesis of NMS.

Table 5.1 Clinical and Biochemical Similarities Between Malignant Hyperpyrexia and the Neuroleptic Malignant Syndrome.

Hyperpyrexia

Hypertonicity of Skeletal Musculature

Tachycardia

Tachypnoea

Hyperkalaemia

Metabolic Acidosis

Elevated Serum Creatine Phosphokinase

Response to Dantrolene Sodium

In some cases, once the symptoms of NMS have resolved, neuroleptic medication has been reintroduced with no recurrence of symptoms (Burke, Fahn, Mayeux, Weinberg, Louis and Willner, 1981; Scarlett, Zimmerman and Berkovic, 1983; Mueller, Vester and Fermaglich, 1983). This observation indicated that individual physiological and environmental factors may be important in NMS, and this was supported by the comparatively well-known occurence of neuroleptic induced heat-stroke in psychiatric patients exposed to high environmental temperatures (Elliot and Brow, 1973; Sarnquist and Larsen, 1973; Mann and Boger, 1978).

The diagnosis of NMS was complicated by the non-drug related syndrome 'acute lethal catatonia' (Feldman, 1984). This syndrome was described well before the introduction of neuroleptic therapy (Stauder, 1934) and can resemble NMS closely (Gelenberg, 1976).

A number of investigators have proposed central mechanisms as the cause of NMS. These include dysfunction of the basal ganglia and possibly of the hypothalamus (Morris, McCormick and Reinarz, 1980), dopamine receptor blockade (Henderson and Wooten, 1981), dopamine depletion (Burke, Fahn, Mayeux, Weinberg, Louis and Willner, 1981) and a combination of inhibition of norepinephrine and dopamine reuptake together with blockade of post-synaptic dopamine receptors (Ansseau, Diricq, Grisor and Collard, 1980). The striking symptomatic likeness between NMS and MH has led other investigators to consider the relationship between these two syndromes. Weinberg and Twersky (1983) proposed that NMS patients should be considered as candidates for developing MH intraoperatively. Other investigators have studied blood samples or muscle biopsy specimens in order to

establish a positive link between NMS and MH. Some of these studies have relied on tests which were not reliable as diagnostic tests for MH. These included the platelet ATP depletion test (Scarlett, Zimmerman and Berkovic, 1983) and a muscle phosphorylase ratio determination (Burke, Fahn, Mayeux, Weinberg, Louis and Willner, 1981). Only three NMS patients have been reported as being tested for MH susceptibility using an in vitro muscle contracture test. Caroff, Rosenberg and Gerber (1983) observed that muscle from their NMS patient developed a contracture of 5g on exposure to 1.2% halothane, but observed no response to caffeine. The diagnosis of this patient was MH positive. Tollefson (1982) reported a NMS patient whose muscle displayed no abnormal responses to halothane or to halothane plus caffeine. Susceptibility to MH was also ruled out in a NMS patient reported by Scarlett, Zimmerman and Berkovic (1983; Moulds, 1983, personal communication). This patient's muscle displayed normal responses to halothane, caffeine, succinylcholine and potassium chloride.

Another feature of NMS which indicated a relationship with MH was the reported efficacy of dantrolene sodium, the muscle relaxant regarded as a specific treatment for MH (Austin and Denborough, 1977; Kolb, Horne and Martz, 1982), in the treatment of NMS. Dantrolene sodium has been used effectively in NMS by both the oral (Coons, Hillman and Marshal, 1982; Delacour, Daoudal, Chapoutot and Rocq, 1981) and the intravenous route (Goekoop and Carbaat, 1982).

5.2 Report of a case of Neuroleptic Malignant Syndrome.

The following case report describes the occurance of NMS in a psychiatric patient reffered to Royal Canberra Hospital, Acton,

A.C.T. The patient, Mr. R.M., was subsequently identified as MHS by *in vitro* muscle contracture testing (Denborough, Collins and Hopkinson, 1984).

Mr R.M. was aged 31 years and was diagnosed as a chronic schizophrenic in October 1982. He was being treated with fluphenazine hydrochloride (5 mg twice a day), fluphenazine decanoate (50 mg intramuscularly once weekly) and benzhexol hydrochloride (2.5 mg twice a day). On the 9th of July, 1983, Mr R.M. complained of nausea and feeling dizzy. He vomited twice, and on the second occasion the vomitus contained coffee-ground material. The patient was admitted to hospital and soon after he had a grand mal fit. His blood pressure was 170/110 and his temperature was 38.5^{o}C. No abnormality was found on clinical examination of the nervous system, and he remained drowsy and febrile for the next two days. His serum creatine phosphokinase was 53,000 units/l (normal range 10 to 150 units/l) and his urine contained myoglobin. A diagnosis of the neuroleptic malignant syndrome was made and his drug treatment was stopped. The patient was treated symptomatically and made an uneventful recovery.

Two months later, Mr R.M. was feeling fit and well, and his mental symptoms had not deteriorated without drug treatment. On clinical examination no abnormality was found and his serum creatine phosphokinase was 87 units/l. *In vitro* muscle tests on the 27th of September, 1983 showed increased muscle contractility diagnostic of susceptibility to malignant hyperpyrexia (Figure 5.1).

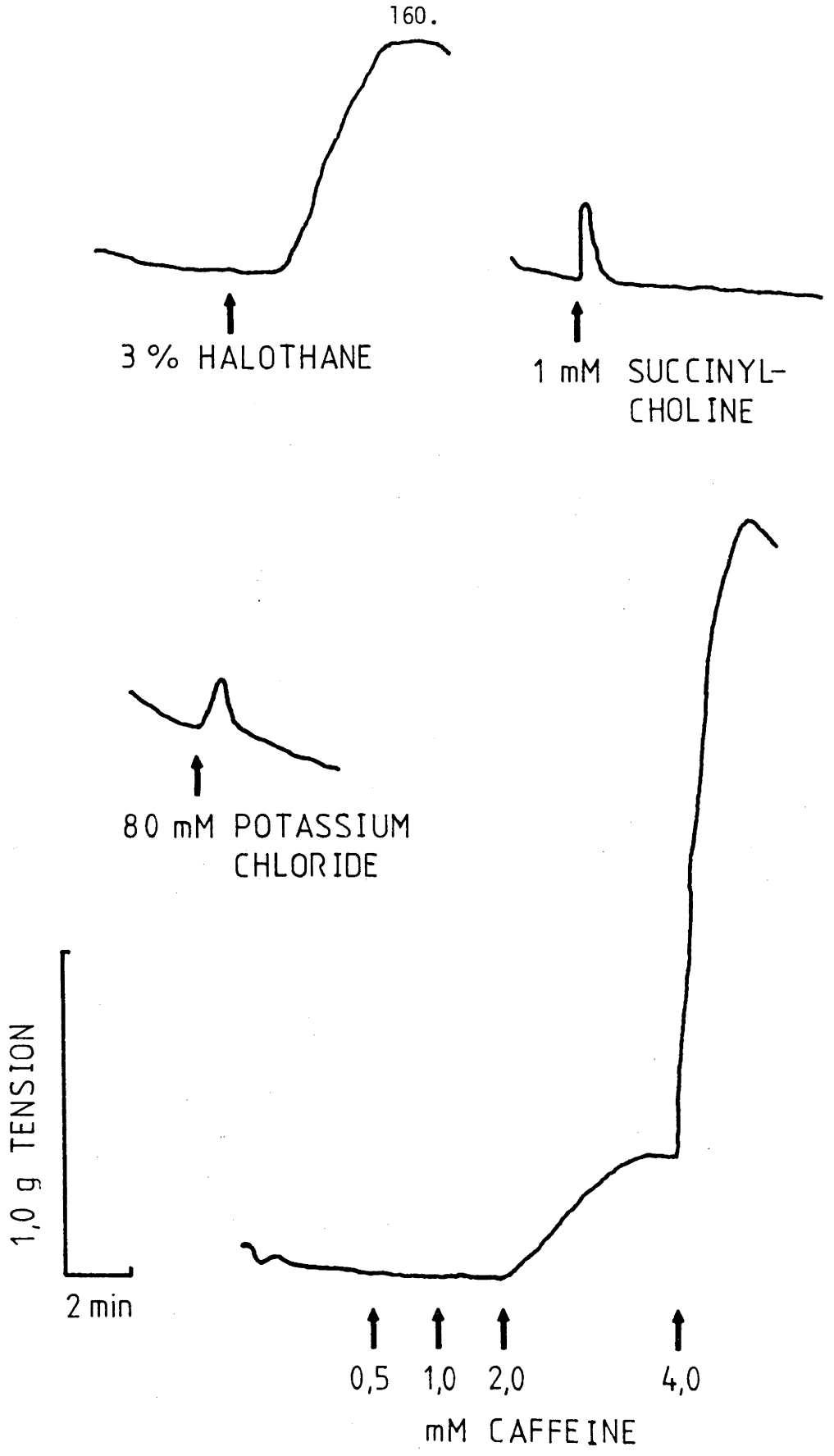


Figure 5.1 Diagnostic Muscle Contracture Testing of Patient R.M. showing Positive Reactions.

The muscle was vastus lateralis.

On the 29th of November, 1983, Mr R.M.'s brother, Mr G.M., was also diagnosed as MHS on *in vitro* muscle testing.

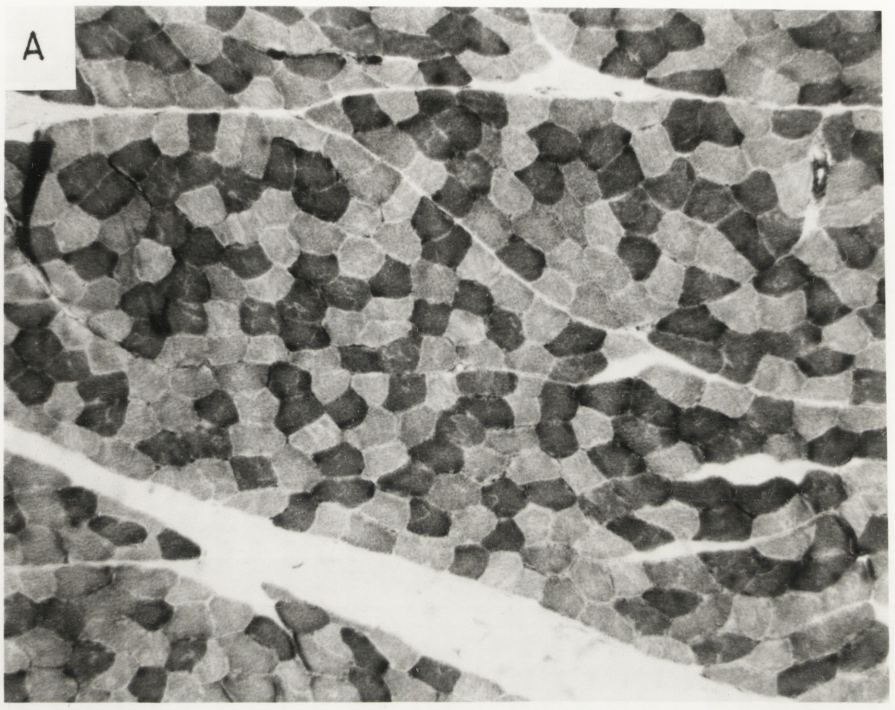
There were no gross abnormalities observed by histological examination of Mr R.M.'s vastus lateralis muscle (Figure 5.2), although the muscle fibres appeared to be slightly larger than normal and displayed some Type I (dark staining) fibre clumping (Hopkinson, 1984, personal communication).

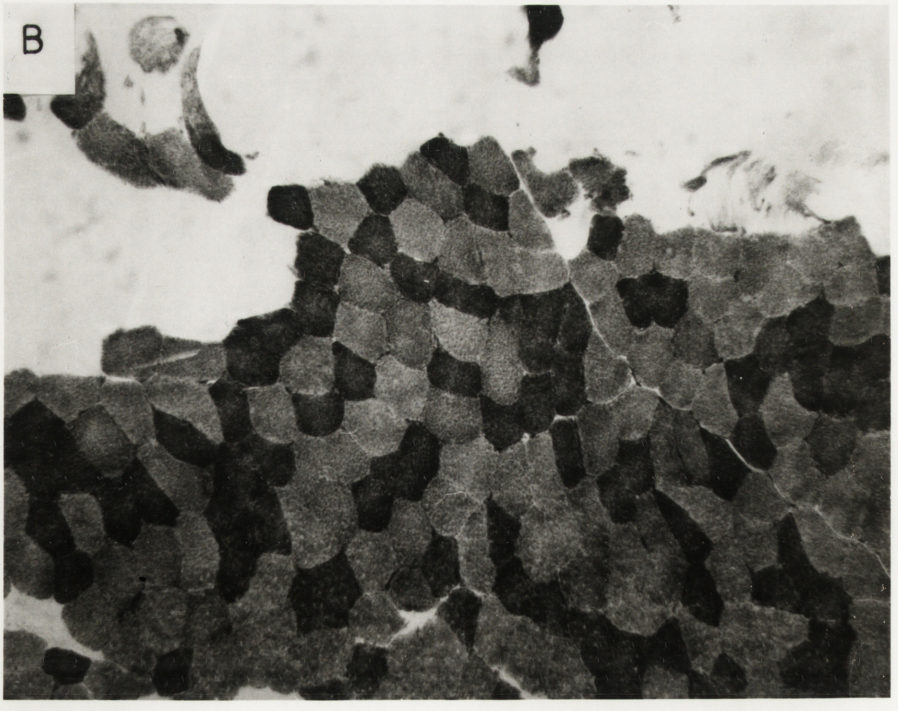
5.3 Discussion

The above case report of a NMS episode in a psychiatric patient and his subsequent identification as MHS supports the theory that NMS and MH are related syndromes. Other reports of *in vitro* skeletal muscle testing of NMS patients have shown that one out of a total of three patients was MHS (positive - Caroff, Rosenberg and Gerber, 1983; negative - Tollefson, 1982 and Scarlett, Zimmerman and Berkovic, 1983). This observation was consistent with the *in vitro* pharmacological finding that neuroleptic drugs acted on both control and MHS porcine skeletal muscle (Chapter 2). These drugs caused contractures in and also potentiated the hypercontractility of MHS muscle. They also caused contracture in control muscle, and induced it to become hypercontractile and behave in a similar way to MHS muscle.

The observations on the efficacy of dantrolene sodium in the treatment of three cases of NMS (Coons, Hillman and Marshal, 1982; Delacour, Daoudal, Chapoutot and Rocq, 1981; Goekoop and Carbaat, 1982) does not necessarily mean these cases were all MHS individuals.

Figure 5.2 Histological Appearance of Vastus Lateralis Muscle of patient R.M. NADH-tetrazolium reductase stain, x 50
(a) normal control (b) Patient R.M.
Micrographs were kindly supplied by Mr K. Hopkinson.


A


B

In vitro pharmacology of the neuroleptic drugs (Chapter 2) has shown that dantrolene sodium had the ability to reverse contractures induced by neuroleptics in both control and MHS porcine muscle. The efficacy of dantrolene sodium in the treatment of MH and NMS may reflect a similar cellular mechanism of precipitation of both these syndromes, that is, high intramyoplasmic Ca^{2+} levels in MH, MH-related NMS and non-MH-related NMS.

The *in vitro* pharmacological effects of neuroleptic drugs were observed at concentrations in the μM range (Chapter 2). The concentration of neuroleptic drug in a patient's serum water, however, has been shown to be in the range 0.1 to 50 nM although this figure was only 5% of the total plasma concentration in the case of CPZ. The other 95% of the drug was bound to plasma proteins (Seeman, 1977; Verbeek, Cardinal, Hill and Midha, 1983). Due to the hydrophobicity of neuroleptic drugs they are very soluble in membranes. In the case of CPZ, an aqueous phase concentration of 10^{-5}M or 10^{-6}M established a membrane phase concentration of 20 mM (that is, 20 mmoles of drug per kg or litre of membrane phase) (Seeman, 1972). Also to be taken into account was the actual concentration of drug present in a typical pharmacological or biochemical experiment. In the case of CPZ, 20-90% of the drug may have been absorbed to the tissue and glassware present (Seeman, 1977). These considerations suggest that the concentration of neuroleptic drugs reached in membranes *in vivo* may have been high enough to produce the effects observed *in vitro*.

The mechanism by which neuroleptic drugs produce an NMS episode may involve many sites of action in both MHS and non-MHS individuals.

The most important of these appeared to be the SR Ca^{2+} regulatory mechanisms of skeletal muscle. Neuroleptic drugs inhibited the uptake of Ca^{2+} from the myoplasm and also caused the release of stored Ca^{2+} from the SR (Chapter 4) thereby raising myoplasmic Ca^{2+} levels. This effect might trigger MH in MHS individuals and also raise Ca^{2+} levels high enough to trigger a MH-like syndrome (that is, NMS) in non-MHS individuals. The observation that dantrolene sodium reversed neuroleptic-induced contractures in vitro (Chapter 2) while being unable to affect either the inhibition of SR Ca^{2+} uptake or acceleration of Ca^{2+} release produced by neuroleptic drugs (Chapter 4) suggested some other site of action. Since dantrolene sodium is thought to act on excitation- contraction coupling (Ellis and Bryant, 1972), neuroleptic drugs may also act at that site. Calmodulin-dependent functions were also inhibited by neuroleptic drugs (Levin and Weiss, 1976).

Another factor contributing to both MH related and non-MH related NMS was the effect of neuroleptics on both the central and peripheral mechanisms of thermoregulation (Kollias and Bullard, 1964). Once hyperpyrexia begins, the inhibition of heat loss mechanisms by these drugs would exacerbate the situation. Individual physiological and environmental conditions were likely to be important in the genesis of a NMS episode. A psychiatric patient receiving neuroleptic therapy who was agitated or restrained, or who was exposed to high environmental temperatures may be at risk.

The drugs PEN and PIM have been shown to differ from the other neuroleptics tested in their in vitro muscle pharmacology (Chapter 2). Unlike the other drugs, PEN and PIM did not induce contracture

in either control or MHS porcine muscle, nor did they induce hypercontractility in control muscle. These observations may have clinical implications if it can be shown that PEN and PIM do not produce NMS *in vivo* in the psychiatric patient population. These drugs were, however, among the most potent calmodulin antagonists studied, and both induced marked effects on the Ca^{2+} transport functions of isolated FSR preparations from both control and MHS porcine muscle. The failure of PEN and PIM to induce *in vitro* contracture may have been due to their insolubility in the aqueous organ bath environment. Pimozide was one of two neuroleptics being taken by a patient who developed NMS, but since the other drug was HPD, no conclusions can be drawn as to PIM's safety (Ansseau, Diricq, Grisar and Collard, 1980). Penfluridol has not been reported in conjunction with NMS. Since both PEN and PIM are recently introduced neuroleptics, more time will be needed to evaluate them in psychiatric practice.

5.4 Summary

Some neuroleptic drugs can trigger MH in susceptible individuals. They are, however, much weaker triggers than halothane or succinylcholine. The psychiatric patient population is exposed to chronic and high dose treatment with neuroleptics and MHS individuals may be at risk. Neuroleptic drugs also appeared to be able to precipitate a MH-like syndrome in non-MHS individuals through their effects on muscle Ca^{2+} regulatory mechanisms.

CHAPTER 6 GENERAL DISCUSSION

The basic abnormality in human and porcine MH is not known, but it is thought to involve a disturbance of myoplasmic Ca^{2+} regulation (Denborough, 1980). Thus, *in vivo*, triggers of MH such as halothane precipitate a rapid and sustained rise in myoplasmic Ca^{2+} levels in MHS skeletal muscle leading to the acute MH reaction. This abnormality of Ca^{2+} regulation in MHS muscle is also observed in *in vitro* pharmacological muscle strip preparations as a hypercontractility to 3% halothane, 2 mM caffeine, 1 mM succinylcholine and 80 mM potassium chloride. The major role in regulating myoplasmic Ca^{2+} concentrations is played by the SR through its properties of uptake, storage and release of Ca^{2+}. These Ca^{2+} storage and transport activities control the contractile activity and much of the metabolism of the muscle fibre. The SR receives the signal to release Ca^{2+}, and therefore initiate contraction, through the E-C coupling mechanism. The details of this mechanism are not known, but it occurs at the triad junction of the SR and the t-tubule where depolarization of the t-tubule membrane stimulates the SR to release Ca^{2+}. It is the E-C coupling mechanism which has been proposed as the site of the basic abnormality in MH skeletal muscle (Austin and Denborough, 1977; Denborough, 1980; Okumura, Crocker and Denborough, 1980). In the present investigation the role of the Ca^{2+}-regulatory protein calmodulin in MH was studied using the porcine MH model. This was achieved by studying the *in vitro* muscle pharmacology and biochemistry of calmodulin antagonist drugs and by studies of isolated calmodulin. It is not known whether calmodulin is involved in E-C coupling, although it has been proposed that

calmodulin plays a role in the excitation-secretion coupling of synaptic transmission in neural tissue (Roufogalis, 1980).

The observations on the in vitro muscle pharmacology and biochemistry of calmodulin antagonist drugs made in the present investigation suggested that the Ca^{2+} regulatory protein calmodulin is not the specific site of action through which these drugs induced changes in the contractile characteristics of control and MHS porcine skeletal muscle. Also, physical and functional studies of calmodulin preparations isolated from control and MHS porcine brain did not show any differences between these proteins. These observations were consistent with other physical (Lorkin and Lehmann, 1983) and functional (Marjanen, Collins and Denborough, 1984) comparisons of control and MHS calmodulins. These findings suggest that calmodulin is not abnormal in porcine MHS tissue, and that calmodulin does not play a role in the hypercontractility of MHS muscle.

In an in vitro pharmacological muscle strip preparation the calmodulin antagonist drugs induced changes in the contractile characteristics of both control and MHS porcine skeletal muscle. The calmodulin antagonist drugs induced contracture in both control and MHS muscle, induced hypercontractility to halothane, caffeine and succinylcholine in control muscle, and also potentiated hypercontractility to halothane, caffeine and succinylcholine in MHS muscle. These changes were consistent with the calmodulin antagonist drugs causing an increase in myoplasmic Ca^{2+} concentration in both control and MHS muscle. This proposal is also consistent with the observed effects of the calmodulin antagonist drugs on isolated FSR preparations. That is, these drugs inhibited the SR Ca^{2+}-dependent

ATPase activity and ATP-dependent Ca^{2+} uptake, and accelerated the efflux of Ca^{2+}, from both control and MHS FSR preparations. Two of the calmodulin antagonist drugs that affected the SR Ca^{2+} transport functions, PEN and PIM, did not affect the in vitro contractile characteristics of control or MHS muscle. This may have been because PEN and PIM were insoluble in the organ bath solution. The activity of PEN and PIM in the biochemical experiments may have been due to the direct exposure of the FSR to the incubating medium containing these drugs.

The calmodulin antagonist drugs used in the present investigation did not provide calmodulin-specific antagonist activity in either the pharmacological muscle strip preparation or the isolated SR membrane preparation. These observations reinforce the growing recognition of the non-specific effects of the calmodulin antagonist drugs which are presently available when used in cellular systems or complex sub-cellular systems, especially in the presence of biological membranes (Corps, Hesketh and Metcalfe, 1982; Roufogalis, Minocherhomjee and Al-Jabore, 1983). The inhibitory activities of the calmodulin antagonist drugs with respect to the SR Ca^{2+}-dependent ATPase activity of both control and MHS porcine skeletal muscle was correlated with the octanol/water partition coefficient of each drug.

The in vitro contractile responses of control and MHS muscle to potassium chloride, in contrast to the responses to halothane, caffeine and succinylcholine, were not affected by the calmodulin antagonist drugs. This observation may have been explained by the ability of calmodulin antagonist drugs to reduce the excitability of excitable membranes such as the sarcolemma (Langslet, 1970; Seeman,

1972). The reason why responses to succinylcholine were not affected by this electrical stabilization of the sarcolemma may have been that succinylcholine interacted with the sarcolemma at a specific membrane receptor (that is, the acetylcholine receptor), while potassium chloride did not. Succinylcholine may have also had a direct effect on the SR (Moulds, 1978). The observation that *in vitro* contractile responses of control and MHS muscle to halothane and caffeine were not affected by the electrical stabilization of the sarcolemma by calmodulin antagonist drugs suggested that halothane and caffeine acted at a site distal to the sarcolemma. Caffeine has been thought to cause *in vitro* contracture by acting directly on the SR causing the release of stored Ca^{2+} (Ogawa, 1970), and this is consistent with these observations. The evidence in favour of the SR as a site of action for halothane has been conflicting, however, and it may be that halothane affects the E-C coupling mechanism (Moulds and Denborough, 1974a; Nelson and Denborough, 1977).

Because of the observations that calmodulin antagonist drugs induced MH-like hypercontractility in control porcine skeletal muscle and potentiated the hypercontractility of MHS muscle *in vitro*, it would be interesting to investigate the *in vivo* effects of these drugs on control and MHS swine. Further experimentation might include an investigation of whether or not calmodulin antagonist drugs trigger a MH episode in MHS swine, and whether or not these drugs induce MH-like symptoms in control swine. Also, the *in vivo* responses of control swine to halothane and succinylcholine after administration of calmodulin antagonist drugs might be studied. Some of these experiments have been fortuitously carried out already at low calmodulin antagonist doses since the premedication which was

administered to both control and MHS swine in the present study (STRESNIL; Smith, Kline and French Laboratories, Australia) contained the butyrophenone azaperone. Azaperone is structurally very similar to the butyrophenone HPD. None of the control or MHS swine reacted adversely to intramuscular injection of 1.5-2 mg/kg azaperone. Higher doses or chronic administration experiments using calmodulin antagonist drugs may have provided different results.

The possible role of calmodulin antagonist drugs in precipitating human MH has attracted recent interest through investigations of NMS, a life-threatening side-effect of neuroleptic therapy (especially using the phenothiazine and butyrophenone drug groups) that closely resembles an MH episode. Observations presented in the present investigation and elsewhere (Caroff, Rosenberg and Gerber, 1983) have shown that chronic, high dose neuroleptic therapy may precipitate a MH episode in MHS individuals. Patients who had experienced a NMS episode and who were diagnosed as MH-negative (Tollefson, 1982; Scarlett, Zimmerman and Berkovic, 1983) may have been affected through the ability of the calmodulin antagonist drugs to raise myoplasmic Ca^{2+} concentrations in normal skeletal muscle and thus mimic the symptoms of MH. Many investigators, however, considered their NMS patients to have undergone a central effect of the calmodulin antagonist drugs involving such mechanisms as dopamine receptor blockade (Henderson and Wooten, 1981), dopamine depletion (Burke, Fahn, Mayeux, Weinberg, Louis and Willner, 1981) and a combination of inhibition of norepinephrine and dopamine reuptake together with blockade of post-synaptic dopamine receptors (Annsseau, Diricq, Grisar and Collard, 1980). The phenothiazine drugs are also extensively used as premedication for surgical procedures involving

halothane anaesthesia (and other volatile inhalational anaesthetic triggers of MH) and succinylcholine. No consistent adverse effects of phenothiazine premedication on anaesthesia have been reported, although isolated cases of hypotension and tachycardia during anaesthesia have been considered to be due to this premedication (Dripps, Vandam, Pierce, Oech and Lurie, 1955; Gold, 1974). A possible role of the phenothiazine drugs (in the form of cough mixture) has also been considered in the Sudden Infant Death Syndrome (SIDS) (Kahn and Blum, 1979). This observation is of interest in the light of the recently described link between MH and the parents of some children who had died from SIDS (Denborough, Galloway and Hopkinson, 1982).

The observation that dantrolene sodium partially reversed contractures induced by calmodulin antagonist drugs in vitro suggested that these drugs were acting, in part, on the E-C coupling mechanism to exert their effects on the contractile characteristics of both control and MHS porcine skeletal muscle. This proposal is consistent with the observations that dantrolene sodium had no effect on the Ca^{2+} transport functions of isolated SR (White, Collins and Denborough, 1983), nor did it effect the changes produced in these functions by the calmodulin antagonist drugs. Also, E-C coupling is thought to be the site of action of dantrolene sodium in skeletal muscle (Ellis and Bryant, 1972). The butyrophenone HPD differed from the phenothiazine calmodulin antagonist drugs in that, although at least as potent as these drugs in inducing changes in the in vitro contractile characteristics of control and MHS muscle, HPD-induced contractures were reversed to a much greater extent by dantrolene sodium (approximately 80%). Trifluoperazine-induced contractures

(representative of the other phenothiazine drugs) were reversed approximately 20% by dantrolene sodium. These observations suggested that HPD induced its in vitro contractile effects mainly through an effect on E-C coupling. This was consistent with the observed effects of HPD on isolated SR Ca^{2+} transport functions, where very high concentrations of HPD were required to induce substantial effects. Another possible site of action of calmodulin antagonist drugs in control and MHS skeletal muscle was troponin C. This Ca^{2+}-binding protein shows extensive sequence homology with calmodulin (Dedman, Jackson, Schreiber and Means, 1978) and has been shown to bind TFP (Levin and Weiss, 1978). This TFP binding to troponin C has been shown to involve Ca^{2+}-binding site III on the troponin C molecule (Klee, Crouch and Richman, 1980). Reid, Gariepy and Hodges (1983) have shown that a synthetic, Ca^{2+}-binding peptide analogue of site III of rabbit skeletal muscle troponin C bound a number of calmodulin antagonist drugs in a phenothiazine selective manner. These authors showed that the butyrophenone HPD had no effect on the circular dichroism spectrum or calcium sensitivity of this apopeptide. Thus, HPD may also differ from TFP in that it did not show appreciable binding to troponin C. These observations suggest that it would be interesting to further examine the effects of HPD on control and MHS muscle. The effects of HPD on E-C coupling might be substantiated, using the in vitro muscle strip preparation, by treatments which preferentially effected E-C coupling such as glycerol or deuterium oxide treatment prior to the addition of HPD (Okumura, Crocker and Denborough, 1980). It might also be interesting to examine the feasability of constructing a HPD or dantrolene sodium affinity column. The passage, and subsequent elution, of partially solubilized protein mixtures obtained from

isolated light, intermediate and heavy SR fractions through such an affinity column might provide some insight into the mechanism of E-C coupling and the location of the dantrolene sodium binding site.

In both control and MHS porcine skeletal muscle calmodulin antagonist drugs caused a disturbance of Ca^{2+} regulation which led to a rise in the myoplasmic Ca^{2+} concentration. When Ca^{2+} regulation of control muscle was disturbed by calmodulin antagonist drugs, the muscle was observed to react to halothane, caffeine and succinylcholine with a MH-like in vitro hypercontractility. These observations supported the hypothesis that the abnormality present in MHS muscle involved a disturbance of Ca^{2+} regulation. The site of this abnormality of Ca^{2+} regulation did not appear to involve the SR Ca^{2+} transport functions since these functions did not differ between control and MHS isolated SR preparations. Among the calmodulin antagonist drugs studied in the present investigation, the hypercontractility of HPD-treated control muscle most closely resembled the hypercontractility of MHS muscle due to its degree of reversal by dantrolene sodium. From the considerations discussed above it appeared that the HPD-induced hypercontractility of control muscle was caused mainly by an effect on the E-C coupling mechanism. Thus, the similarities between MHS muscle and HPD-treated control muscle implicated the E-C coupling mechanism as the site of the basic abnormality in porcine MH.

The present investigation into the role of the Ca^{2+} regulatory protein calmodulin in porcine MH has led to the following major conclusions:

- calmodulin was not abnormal in porcine MHS tissue

- the calmodulin antagonist drugs used in the present investigation were neither specific nor selective for calmodulin when used in the pharmacological muscle strip preparation or the isolated SR membrane preparation
- the calmodulin antagonist drugs used in neuroleptic therapy (especially the phenothiazine and butyrophenone groups) may precipitate a MH episode in MHS individuals
- the site of the basic abnormality in porcine MHS skeletal muscle appears to involve the E-C coupling mechanism.

Bibliography

Akera, T. and Brody, T.M. (1969) The interaction between chlorpromazine free radical and microsomal sodium- and potassium-activated adenosine triphosphatase from rat brain.
Mol. Pharmacol., 5, 605-614.

Akera, T. and Brody, T.M. (1972) Effects of chlorpromazine free radical on brain microsomal enzymes.
Biochem. Pharmacol., 21, 1403-1411.

Aldrete, A. (1981) Advances in the diagnosis and Treatment of Malignant Hyperthermia.
Acta Anaesth. Scand., 25, 477-483.

Allen, R.N. and White, H.C. (1972) Side effects of parenteral long-acting phenothiazines.
Brit. Med. J., 1, 221.

Amaranath, L., Lavin, T.J., Trusso, R.A. and Boutros, A.R. (1983) Evaluation of creatine phosphokinase screening as a predictor of malignant hyperthermia.
Br. J. Anaesth., 55, 531-533.

Andersson, K.E. (1972) Effects of chlorpromazine, imipramine and quinidine on the mechanical activity of single skeletal muscle fibres of the frog.
Acta Physiol. Scand., 85, 532-546.

Ansseau, M., Diricq, S., Grisar, T. and Collard, J. (1980) Biochemical and neuroendocrine approaches to a malignant syndrome of neuroleptics.
Acta Psychiat. Belg., 80, 600-606.

Ashley, C.C. (1978) Calcium ion regulation in barnacle muscle fibres and its relation to force development.
Ann. N.Y. Acad. Sci., 307, 308-329.

Austin, K.L. and Denborough, M.A. (1977) Drug Treatment of Malignant Hyperpyrexia.
Anaesth. Int. Care, 5, 207-213.

Balzer, H. and Hellenbrecht, D. (1969) Beeinflussung des calcium-austauschs und der muskelfunktion des M. rectus und sartorius des frosches dur chlorpromazin, prenylamin, imipramin und reserpin.
Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak., 264, 129-146.

Balzer, H., Makinose, M. and Hasselbach, W. (1968) The inhibition of the sarcoplasmic calcium pump by prenylamine, reserpine, chlorpromazine and imipramine.
Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak., 260, 444-455.

Berman, M.C. and Kench, J.E. (1973) Biochemical features of malignant hyperthermia in Landrace pigs. In International Symposium of Malignant Hyperthermia, Gordon, R.A., Britt, B.A. and Kalow, W. (eds), pp 287-297. C.C. Thomas, Springfield, Illinois.

Blanck, T.J.J. and Gruener, R.P. (1983) Malignant Hyperpyrexia
Biochem. Pharmacol., 32, 2287-2289.

Blanck, T.J.J., Gruener, R., Suffecool, S.L. and Thompson, M. (1981) Calcium uptake by isolated sarcoplasmic reticulum: examination of halothane inhibition, pH dependence and Ca^{2+} dependence of normal and malignant hyperthermic human muscle.
Anaesth. Analg., 60, 492-498.

Bleichner, G., Squara, P. and Parent, A. (1981) Hypertonia and malignant hyperthermia due to morphine and neuroleptic.
Lancet, i, 386-387.

Bloom, W. and Fawcett, D.W. (1975) A Textbook of Histology. W.B. Saunders Company, Philadelphia.

Bourne, G.H. (1972) The Structure and Function of Muscle. Vol. I, Academic Press, New York.

Boynton, A.L., Whitfield, J.F. and MacManus, J.P. (1980) Calmodulin stimulates DNA synthesis by rat liver cells. Biochem. Biophys. Res. Commun., 95, 745-749.

Bradley, W.G., Ward, M.,Murchison, D., Hall, L. and Woolf, N. (1973) Clinical, electrophysiological and pathological studies on malignant hyperpyrexia. Proc. R. Soc. Med. (Lond.), 66, 67-68.

Briskey, E.J. (1964) Etiological status and associated studies of pale, soft, exudative porcine musculature. In Advances in Food Research. Vol. 13, pp 89-178. Chichester, C.O., Mrak, E.M. and Stewart, G.F. (eds). Academic Press, New York.

Britt, B.A. (1977) Malignant hyperthermia: aetiology and pathophysiology. In Anaesthesiology; Proceedings of the World Congress of Anaesthesiology, Hulsz, E., Sanchez-Hernandex, J.A., Vasconcelos, G. and Lunn, J.N. (eds), pp 439-451. Excerpta Medica, Amsterdam.

Britt, B.A. (1979) Etiology and pathophysiology of malignant hyperthermia. Fed. Proc., 38, 44-48.

Britt, B.A. (1983) Malignant Hyperthermia. In Complications in Anaesthesiology, Orkin, F.K. and Cooperman, L.H. (eds), pp 291-313. J.B. Lippincott Co., Philadelphia, U.S.A.

Britt, B.A. Endrenyi, L. and Cadman, D.L. (1975) Calcium uptake into muscle of pigs susceptible to malignant hyperthermia: in vitro and in vivo studies with and without halothane. Br. J. Anaesth., 47, 650-653.

Britt, B.A., Endrenyi, L. Kalow, W. and Peters, P.L. (1976) The Adenosine triphosphate (ATP) depletion test: comparison with the caffeine contracture test as a method of diagnosing malignant hyperthermia susceptibility.
Can. Anaesth. Soc. J., 23, 624-635.

Britt, B.A., Endrenyi, L., Peters, P.L., Kwong, W. and Kadijevic, L. (1976) Screening of Malignant Hyperthermia susceptible families by creatine phosphokinase measurement and other clinical investigations.
Can. Anaesth. Soc. J., 23, 263-284.

Britt, B.A., Kalow, W., Gordon, A., Humphrey, J.C. and Rewcastle, N.B. (1973) Malignant hyperthermia: an investigation of five patients.
Can. Anaesth. Soc. J., 20, 431-467.

Brucker, R.F., Williams, C.H.,Popinigis, J., Galvez, T.L., Vail, W.J. and Taylor, C.A. (1973) In vitro studies on liver mitochondria and skeletal muscle sarcoplasmic reticulum fragments isolated from hyperpyrexic swine. In International Symposium on Malignant Hyperthermia, Gordon, R.A., Britt, B.A. and Kalow, W. (eds), pp 238-270. C.C. Thomas, Springfield, Illinois.

Burke, R.E., Fahn, S., Mayeux, R., Weinberg, H., Louis, K. and Willner, J.H. (1981) Neuroleptic malignant syndrome caused by dopamine-depleting drugs in a patient with Huntington disease.
Neurology (Ny), 31, 1022-1026.

Butler, T.M., Siegman, M.J., Mooers, S.U. and Barsotti, R.J. (1983) Myosin light chain phosphorylation does not modulate cross-bridge cycling rate in mouse skeletal muscle.
Science, 220, 1167-1169.

Campbell, I.T., Ellis, F.R., Halsall, P.J. and Hogge, M. St. J. (1982) Anthropometric studies of human subjects susceptible to malignant hyperpyrexia. Acta. Anaesth. Scand., 26, 363-367.

Campbell, K.P. and MacLennan, D.H. (1981) Purification and characterization of the 53,000-dalton glycoprotein from the sarcoplasmic reticulum.
J. Biol. Chem., 256, 4626-4632.

Campbell, K.P. and MacLennan, D.H. (1982) A calmodulin-dependent protein kinase system from skeletal muscle sarcoplasmic reticulum.
J. Biol. Chem., 257, 1238-1246.

Caroff, S.N. (1980) The neuroleptic malignant syndrome.
J. Clin. Psychiatry, 41, 79-83.

Caroff, S., Rosenberg, H. and Gerber, J.C. (1983) Neuroleptic malignant syndrome and malignant hyperthermia.
Lancet, i, 244.

Cheah, K.S. and Cheah, A.M. (1978) Calcium movements in skeletal muscle mitochondria of malignant hyperthermic pigs.
Febs Letters, 95, 307-310.

Cheah, K.S. and Cheah, A.M. (1979) Mitochondrial calcium efflux and porcine stress-susceptibility.
Experientia, 35, 1001-1003.

Cheung, W.Y. (1980) Calmodulin plays a pivotal role in cellular regulation.
Science, 207, 19-27.

Cheung, W.Y., Lynch, T.J. and Wallace, R.W. (1978) An endogenous Ca^{2+}-dependent activator protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase.
Adv. Cyclic Nucl. Res., 9, 233-251.

Chiesi, M. and Carafoli, E. (1982) The regulation of Ca^{2+} transport by fast skeletal muscle sarcoplasmic reticulum.
J. Biol. Chem., 257, 984-991.

Chiesi, M. and Carafoli, E. (1983) Role of Calmodulin in skeletal muscle sarcoplasmic reticulum.
Biochemistry, 22, 985-993.

Clayberger, C., Goodman, D.B.P. and Rasmussen, H. (1981) Regulation of cAMP metabolism in the rat erythrocyte during chronic adrenergic stimulation. Evidence for calmodulin-mediated alteration of membrane bound phosphodiesterase activity.
J. Membr. Biol., 58, 191-201.

Cohen,C., Caspar, D.L.D., Parry, D.A.D. and Lucas, R.M. (1971) Tropomyosin crystal dynamics.
Cold Spring Harbour Symposium on Quantitative Biology, 36, 205-216.

Cohen, P., Burchell, A., Foulkes, J.G., Cohen, P.T.W., Vanaman, T.C. and Nairn, A.C. (1978) Identification of the Ca^{2+}-dependent modulator protein as the fourth subunit of rabbit skeletal muscle phosphorylase kinase.
Febs Letters, 92, 287-293.

Coons, D.J., Hillman, F.J. and Marshal, R.W. (1982) Treatment of neuroleptic malignant syndrome with dantrolene sodium: a case report.
Am. J. Psychiatry, 139, 944-945.

Cooper, R.A. (1979) Heat and neuroleptics: a deadly combination.
Am. J. Psychiatry, 136, 466-467.

Corps, A.N., Hesketh, T.R. and Metcalfe, J.C. (1982) Limitations on the use of phenothiazines and local anaesthetics as indicators of calmodulin function in intact cells.
Febs Letters, 138, 280-284.

Costantin, L.L. (1975) Contractile activation in skeletal muscle. Prog. Biophys. Molec. Biol., 29, 197-224.

Crouch, T.H., Holroyde, M.J., Collins, J.H., Solaro, R.J. and Potter, J.D. (1981) Interaction of calmodulin with skeletal muscle myosin light chain kinase. Biochemistry, 20, 6318-6325.

Crouch, T.H. and Klee, C.B. (1980) Positive co-operative binding of calcium to bovine brain calmodulin. Biochemistry, 19, 3692-3698.

Crow, M.T. and Kushnerick, M.J. (1982) Phosphorylation of myosin light chains in mouse fast-twitch muscle associated with reduced actomyosin turnover. Science, 217, 835-837.

Dedman, J.R., Brinkley, B.R. and Means, A.R. (1979) Regulation of microfilaments and microtubules by calcium and cyclic AMP. Adv. Cyclic Nucl. Res., 11, 131-174.

Dedman, J.R., Jackson, R.L., Schreiber, W.E. and Means, A.R. (1978) Sequence homology of the Ca^{2+}-dependent regulator of cyclic nucleotide phosphodiesterase from rat testis with other Ca^{2+}-binding proteins. J. Biol. Chem., 253, 343-346.

De Jong, R.H., Heavner, J.E. and Amory, D.W. (1974) Malignant hyperpyrexia in the cat. Anaesthesiology, 41, 608-609.

Delacour, J.L., Daoudal, P., Chapoutot, J.L. and Rocq, B. (1981) Traitment du syndrome malin des neuroleptiques par le dantrolene. Nouv. Presse Med., 10, 3572.

Delay, J., Pichot, P. and Lemperiere, T. (1960) Un neuroleptique majeur non phenothiazine et non reserpinique l'haloperidol dans le traitement des psychoses. Ann. Med. Psychol., 118, 145-152.

Denborough, M.A. and Lovell, R.R.H. (1960) Anaesthetic deaths in a family.
Lancet, ii, 45.

Denborough, M.A., Warne, G.L., Moulds, R.F.W., Tse, P. and Martin, F.I.R. (1974) Insulin secretion in Malignant Hyperpyrexia.
Br. Med. J., 3, 493-495.

Desmedt, J.E. and Hainaut, K. (1977) Inhibition of the intracellular release of calcium by dantrolene in barnacle giant muscle fibres.
J. Physiol., 265, 565-585.

Desmedt, J.E. and Hainaut, K. (1979) Dantrolene and A23187 ionophore: specific action on calcium channels revealed by the aquorin method.
Biochem. Pharmacol., 28, 957-964.

Dhalla, N.S., Sulakhe, P.V., Clinch, N.F., Wade, J.G. and Naimark, A. (1972) Influence of fluothane on calcium accumulation by the heavy microsomal fraction of human skeletal muscle: comparison with a patient with malignant hyperpyrexia.
Biochem. Med., 6, 333-343.

Douglas, W.W. and Rubin, R.P. (1963) The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling.
J. Physiol. Lond. 167, 288-310.

Dripps, R.D., Vandam, L.D., Pierce, E.C., Oech, S.R. and Lurie, A.A. (1955) The use of chlorpromazine in anaesthesia and surgery.
Ann. Surgery, 142, 774-785.

Drury, P.M.E. and Gilbertson, A.A. (1970) Malignant Hyperpyrexia and anaesthesia.
Brit. J. Anaesth., 42, 1021.

Ebashi, S. (1980) Regulation of muscle contraction.
Proc. Royal Soc. London B, 207, 259-286.

Ebashi, S., Kodama, A. and Ebashi, F. (1968) Troponin. I. Preparation and physiological function.
J. Biochem., 64, 465-477.

Elliot, A. and Offer, G. (1978) Shape and flexibility of the myosin molecule.
J. Molec. Biol., 125, 505.

Elliott, C.F. and Broe, G.A. (1973) Hyperpyrexia and phenothiazine medication.
Med. J. Aust., 1, 462.

Ellis, F.R., Keaney, N.P., Harriman, D.G.F., Sumner, D.W., Kyei-Mensah, K., Tyrrell, J.H., Hargreaves, J.B., Parikh, R.K. and Mulrooney, P.L. (1972) Screening for malignant hyperpyrexia.
Br. Med. J., 3, 559-561.

Ellis, K.O. and Bryant, S.H. (1972) Excitation-Contraction Uncoupling in Skeletal Muscle by Dantrolene Sodium.
Naunyn-Schmiedeberg's Arch. Pharmacol., 274, 107-109.

Ellis, K.O., Castellion, A.W., Honkomp, L.H., Wessels, F.L., Carpenter J.F. and Halliday, R.P. (1973) Dantrolene, a direct acting skeletal muscle relaxant.
J. Pharm. Sci., 62, 948-951.

Feibel, J.H. and Schiffer, R.B. (1981) Sympathoadreno-medullary hyperactivity in the neuroleptic malignant syndrome: A case report.
Am. J. Psychiatry, 138, 1115-1116.

Feldman, J. (1984) Neuroleptic malignant syndrome.
J. Am. Med. Assn., 251, 727.

Fiehn, W. and Hasselbach, W. (1969) The effect of diethyl-ether upon the function of the vesicles of sarcoplasmic reticulum.
Eur. J. Biochem., 9, 574-578.

Fletcher, R., Blennow, G., Olsson, A.-K., Ranklev, E. and Tornebrandt, K. (1982) Malignant Hyperthermia in a Myopathic Child. Prolonged Postoperative Course Requiring Dantrolene.
Acta. Anaesth. Scand., 26, 435-438.

Frank, G.B. (1982) Roles of extracellular and "trigger" calcium ion in excitation-contraction coupling in skeletal muscle.
Can. J. Physiol. Pharmacol., 60, 427-439.

Franzini-Armstrong, C. (1980) Structure of sarcoplasmic reticulum.
Fed. Proc., 39, 2403-2409.

Freed, E. (1981) Alcohol-triggered-neuroleptic-induced tremor, rigidity and dystonia.
Med. J. Aust., 2, 44-45.

Frisk-Holmberg, M. and van der Kleijn, E. (1972) The relationship between the lipophilic nature of tricyclic neuroleptics and antidepressants, and histamine release.
Eur. J. Pharmacol., 18, 139-147.

Gallant, E.M., Godt, R.E. and Gronert, G.A. (1979) Role of plasma membrane defect of skeletal muscle in malignant hyperthermia.
Muscle and Nerve, 2, 491-494.

Gelenberg, A.J. (1976) The catatonic syndrome
Lancet, i, 1339-1341.

Gietzen, K., Wuthrich, A. and Bader, H. (1981) R24571: a new powerful inhibitor of red blood cell Ca^{2+}-transport ATPase and of calmodulin-regulated functions.

Biochem. Biophys. Res. Commun., 101, 418-425.

Giger, U. and Kaplan, R.F. (1983) Halothane-induced ATP Depletion in Platelets from Patients Susceptible to Malignant Hyperthermia and Controls.
Anesthesiology, 58, 347-352.

Gil, M.G., Alemany, S., Cao, D.M., Castano, J.G. and Mato, J.M. (1980) Calmodulin modulates phospholipid methylation in Dictyostelium discoideum. Biochem. Biophys. Res. Commun., 94, 1325-1330.

Goekoop, J.G. and Carbaat, P.A.T. (1982) Treatment of neuroleptic malignant syndrome with dantrolene.
Lancet, ii, 49-50.

Gold, M.I. (1974) Profound hypotension associated with preoperative use of phenothiazines.
Anesth. Analg., 53, 844-848.

Grand, R.J.A., Nairn, A.C. and Perry, S.V. (1980) The preparation of calmodulins from barley (Hordeum sp.) and basidiomycete fungi.
Biochem. J., 185, 755-760.

Greaser, M.L., Cassens, R.G., Hoekstra, W.G. and Briskey, E.J. (1969) Effects of diethylether and thymol on the ultrastructural and biochemical properties of purified sarcoplasmic reticulum fragments from skeletal muscle.
Biochim. Biophys. Acta, 193, 73-81.

Gronert, G.A., Heffron, J.J.A. and Taylor, S.R. (1979) Skeletal muscle sarcoplasmic reticulum in porcine malignant hyperthermia.
Eur. J. Pharmacol., 58, 179-187.

Gronert, G.A. and Theye, R.A. (1976) Halothane-induced porcine malignant hyperthermia: metabolic and haemodynamic changes.
Anesthesiology, 44, 36-43.

Gronert, G.A. and Van Dyke, R.A. (1984) Phosphorylase activity and malignant hyperthermia.
Anesth. Analg., 63, 89.

Guth, P.S. and Spirtes, M.A. (1964) The phenothiazine tranquilizers: biochemical and biophysical actions.
Int. Rev. Neurobiol., 7, 231-278.

Hall, L.W., Woolf, N. Bradley, J.W.P. and Jolly, D.W. (1966) Unusual reaction to suxamethonium chloride.
Br. Med. J., 2, 1305.

Halsall, P.J. and Ellis, F.R. (1979) A screening test for the malignant hyperpyrexia phenotype using suxamethonium-induced contracture of muscle treated with caffeine, and its inhibition by dantrolene.
Br. Med. J., 3, 454-456.

Hanbauer, I., Pradham, S. and Yang, H.T.Y. (1980) Role of calmodulin in dopaminergic transmission.
Ann. N.Y. Acad. Sci., 356, 292-303.

Harper, J.R., Cheung, W.Y., Wallace, R.W., Levine, S.N. and Steiner, A.L. (1980) In Calcium and Cell Function, W.Y. Cheung (ed), Vol. 1, pp 273-290. Academic Press, New York.

Harriman, D.G.F. (1982) The pathology of malignant hyperpyrexia. In Skeletal Muscle Pathology. F.L. Mastaglia and J. Walton, eds, pp 575-591. Churchill Livingstone, Melbourne.

Harrison, G.G., Saunders, S.J., Biebuyck, J.F., Hickman, R., Dent, D.M., Weaver, V. and Terblanche, J. (1969) Anaesthetic-induced malignant hyperpyrexia and a method for its prediction.
Brit. J. Anaesth., 41, 844-855.

Heilbrunn, L.V. (1943) An Outline of General Physiology, 2nd ed. W.B. Saunders Co., Philadelphia, PA, U.S.A.

Heinonen, J.K. and Lahti, R.J. (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal. Biochem., 113, 313-317.

Henderson, V.W. and Wooten, G.F. (1981) Neuroleptic malignant syndrome: a pathogenic role for dopamine receptor blockade. Neurology (Ny), 31, 132-137.

Hidaka, H., Yamaki, T., Naka, M., Tanaka, T., Hayashi, H., and Kabayashi, R. (1980) Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. Mol. Pharmacol., 17, 66-72.

Hidaka, H., Yamaki, T., Totsuka, T. and Asano, M. (1979) Selective inhibitors of Ca^{2+}-binding modulator of phosphodiesterase produce vascular relaxation and inhibit actin-myosin interaction. Mol. Pharmacol., 15, 49-59.

Hinchen, J.D. (1969) Practical Statistics for Chemical Research. Metheun and Company and Scientific Paperbacks, London.

Ho, M.-M., Scales, D.J. and Inesi, G. (1983) The effect of trifluoperazine on the sarcoplasmic reticulum membrane. Biochim. Biophys. Acta, 730, 64-70.

Huxley, A.F. and Niedergerke, R. (1954) Interference microscopy of living muscle fibres. Nature, 173, 971-973.

Huxley, H.E. (1962) In Muscle as a Tissue, p. 63. Rodahl, K. and Horvath S.M. (eds).
McGraw-Hill Book Co., New York.

Huxley, H.E. (1963) Electron-microscopic studies on the structure of natural and synthetic protein filaments from striated muscle.
J. Molec. Biol., 7, 281-308.

Huxley, H.E. and Brown, W. (1967) The low angle X-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor.
J. Molec. Biol., 30, 383-434.

Huxley, H.E. and Hanson, J. (1954) Changes in the cross striations of muscle during contraction and stretch and their structural interpretation.
Nature, 173, 973-976.

Ilundain, A. and Naftalin, R.J. (1979) Role of Ca^{2+}-dependent regulator protein in intestinal secretion.
Nature, 279, 446-448.

Iqbal, Z. and Ochs, S. (1980) Calmodulin in mammalian nerve.
J. Neurobiol., 11, 311-318.

Isaacs, H. and Barlow, M.B. (1970) The genetic background to malignant hyperpyrexia revealed by serum creatine phosphokinase estimations in asymptomatic relatives.
Br. J. Anaesth., 42, 1007-1084.

Isaacs, H., Heffron, J.J.A. and Badenhorst, M. (1975) Predictive tests for malignant hyperpyrexia.
Br. J. Anaesth., 47, 1075-1080.

Jamieson, G.A. Jr., Vanaman, T.C. and Blum, J.J. (1979) Presence of calmodulin in Tetrahymena.
Proc. Nat. Acad. Sci. (USA), 76, 6471-6475.

Jordon, O.M., Barak, A.J., Noffsinger, J.K., Chapin, J. and Wingard, D.W. (1980) Phospholipid abnormalities in sarcoplasmic reticulum from malignant hyperthermic swine.
I.R.C.S. Med. Sci., 8, 618-619.

Kahn, A. and Blum, D. (1979) Possible role of phenothiazines in sudden infant death.
Lancet, ii, 364-365.

Kakiuchi, S., Sobue, K., Yamazaki, R., Kambayashi, J., Sakon, M. and Kosaki, G. (1981) Lack of tissue specificity of calmodulin: a rapid and high-yield purification method.
Febs Letters, 126, 203-207.

Kalow, W., Britt, B.A., Terreau, M.E. and Haist, C. (1970) Metabolic error of muscle metabolism after recovery from Malignant Hyperthermia.
Lancet, ii, 895-898.

Katz, S. and Remtulla, M.A. (1978) Phosphodiesterase protein activator stimulates calcium transport in cardiac microsomal preparations enriched in sarcoplasmic reticulum.
Biochem. Biophys. Res. Commun., 83, 1373-1379.

Kelfer, H.M., Singer, W.D. and Reynolds, R.N. (1983) Malignant Hyperthermia in a Child with Duchenne Muscular Dystrophy.
Pediatrics, 71, 118-119.

King, J.O. and Denborough, M.A. (1973) Anaesthetic-induced malignant hyperpyrexia in children.
J. Paediat., 83, 37-40.

King, J.O., Denborough, M.A. and Zapf, P.W. (1972) Inheritance of Malignant Hyperpyrexia.
Lancet, i, 365-370.

Klee, C.B. (1977) Conformational transition accompanying the binding of Ca^{2+} to the protein activator of 3'5'-cyclic adenosine monophosphate phosphodiesterase.
Biochemistry, 16, 1017-1024.

Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Calmodulin.
Ann. Rev. Biochem., 49, 489-515.

Kolb, M.E., Horne, M.L. and Martz, R. (1982) Dantrolene in Human Malignant Hyperthermia.
Anesthesiology, 56, 254-262.

Kollias, J. and Bullard, R.W. (1964) The influence of chlorpromazine on physical and chemical mechanisms of temperature regulation in the rat.
J. Pharmacol. Exp. Ther., 145, 373-381.

Kretsinger, R.H. (1980) Structure and evolution of calcium-modulated proteins.
CRC Crit. Rev. Biochem., 7, 119-174.

Kretsinger, R.H. and Barry, C.D. (1975) The predicted structure of the calcium-binding component of troponin.
Biochim. Biophys. Acta, 405, 40-52.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
Nature, 227, 680-685.

Landon, D.N. (1982) Skeletal muscle - normal morphology, development and innervation. In Skeletal Muscle Pathology, pp 1-87, F.L. Mastaglia and J. Walton, (eds). Churchill Livingstone, Melbourne.

Langslet, A. (1970) Membrane stabilization and cardiac effects of d, l-propranolol, d-propranolol and chlorpromazine.
Eur. J. Pharmacol., 13, 6-14.

La Porte, D.C., Wierman, B.M. and Storm, D.R. (1980) Calcium-induced exposure of a hydrophobic surface on calmodulin.
Biochemistry, 19, 3814-3819.

Lau, Y.H., Caswell, A.H. and Brunschwig, J.-P (1977) Isolation of transverse tubules by fractionation of triad junctions of skeletal muscle.
J. Biol. Chem., 252, 5565-5574.

Levin, R.M. and Weiss, B. (1976) Mechanism by which psychotropic drugs inhibit adenosine cyclic 3'5'-monophosphate phosphodiesterase in brain.
Mol. Pharmacol., 12, 581-589.

Levin, R.M. and Weiss, B. (1977) Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase.
Mol. Pharmacol., 13, 690-697.

Levin, R.M. and Weiss, B. (1978) Specificity of the binding of trifluoperazine to the calcium-dependent activator of phosphodiesterase and to a series of other calcium binding proteins.
Biochim. Biophys. Acta, 540, 197-204.

Levin, R.M. and Weiss, B. (1979) Selective binding of antipsychotic and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase.
J. Pharmacol. Exp. Ther., 208, 454-459.

Lin, Y.M., Liu, Y.P. and Cheung, W.Y. (1974) Purification and characterization of a protein activator of cyclic nucleotide phosphodiesterase from bovine brain.
Methods in Enzymol., 38, 262-273.

Lister, D., Hall, G.M. and Lucke, J.N. (1974) Catecholamines in suxamethonium-induced malignant hyperthermia in Pietrain pigs. Br. J. Anaesth., 46, 803-804.

Lorkin, P.A. and Lehmann, H. (1983a) Investigation of malignant hyperthermia: analysis of skeletal muscle proteins from normal and halothane sensitive pigs by two dimensional gel electrophoresis. J. Med. Genet., 20, 18-24.

Lorkin, P.A. and Lehmann, H. (1983b) Malignant hyperthermia in pigs: a search for abnormalities in Ca^{2+} binding proteins. Febs Letters, 153, 81-87.

Louis, C.F., Turnquist, J. and Jarvis, B. (1983) Inhibition of calmodulin-dependent and independent cardiac sarcoplasmic reticulum activities by R24571. Cell Calcium, 4, 107-116.

Lutsky, I. Witkowski, J. and Henschel, E.O. (1982) HLA typing in a family prone to malignant hyperthermia. Anesthesiology, 56, 224-226.

Lydiatt, J.S. and Hill, G.E. (1981) Treatment of Heatstroke with dantrolene. J. Am. Med. Assn., 246, 41-42.

Mann, S.C. and Boger, W.P. (1978) Psychotropic drugs, summer heat and humidity, and hyperpyrexia: a danger restated. Am. J. Psychiatry, 135, 1097-1100.

Manning, D.R. and Stull, J.T. (1979) Myosin light chain phosphorylation and phosphorylase a activity in rat extensor digitorum longus muscle. Biochem. Biophys. Res. Commun., 90, 164-170.

Marjanen, L.A., Collins, S.P. and Denborough, M.A. (1984) Calmodulin and Malignant Hyperpyrexia.
Biochem. Med., in press.

Marjanen, L.A., Shaw, D.C. and Denborough, M.A. (1983) Comparison of adenylate kinase from normal and malignant hyperpyrexic porcine muscle.
Biochem. Med., 29, 164-170.

Martonosi, A.N. (1982) Transport of Calcium by sarcoplasmic reticulum. In Calcium and Cell Function, pp 37-102, W.Y. Cheung (ed). Academic Press, New York.

Mathias, R., Levis, R. and Eixenberg, R. (1980) Electrical models of excitation-contraction coupling and charge movement in skeletal muscle.
J. Gen. Physiol., 76, 1-31.

Mayr, G.W. and Heilmeyer, L.M.G. (1983) Skeletal muscle myosin light chain kinase. A refined structural model.
Febs Letters, 157, 225-231.

McIntosh, D.B., Berman, M.C. and Kench, J.E. (1977) Characteristics of sarcoplasmic reticulum from slowly glycolysing and from rapidly glycolysing pig skeletal muscle post mortem.
Biochem. J., 166, 387-398.

McPherson, E. and Taylor, C.A., Jr. (1982) The Genetics of Malignant Hyperthermia: Evidence for Heterogeneity.
Am. J. Med. Genet., 11, 273-285.

Michalak, M., Campbell, K.P. and MacLennan, D.H. (1980) Localization of the high affinity calcium binding protein and an intrinsic glycoprotein in sarcoplasmic reticulum membranes.
J. Biol. Chem., 255, 1317-1326.

Morris, H.H., McCormick, W.F. and Reinarz, J.A. (1980) Neuroleptic malignant syndrome.
Arch. Neurol., 37, 462-463.

Moulds, R.F.W. (1978) The site of the abnormality in MH muscle: a comparison of MH muscle and denervated muscle. In Malignant Hyperthermia, pp 49-65, Aldrete, J.A. and Britt, B.A. (eds). Grune and Stratton, New York.

Moulds, R.F.W. and Denborough, M.A. (1974a) A study of the action of caffeine, halothane, potassium chloride and procaine on normal human skeletal muscle.
Clin. Exp. Pharmacol. Physiol., 1, 197-209.

Moulds, R.F.W. and Denborough, M.A. (1974b) Biochemical basis of malignant hyperpyrexia.
Br. Med. J., 2, 241-244.

Moulds, R.F.W. and Denborough, M.A. (1974c) Identification of susceptibility to malignant hyperpyrexia.
Br. Med. J., 2, 245-247.

Moulds, R.F.W. and Denborough, M.A. (1974d) Myopathies and malignant hyperpyrexia.
Br. Med. J., 3, 520.

Mueller, P.S., Vester, J.W. and Fermaglich, J. (1983) Neuroleptic malignant syndrome. Successful treatment with bromocriptine.
J. Am. Med. Assn., 249, 386-388.

Nastuk, W.L. and Hodgkin, A.L. (1950) The electrical activity of single muscle fibres.
J. Cell. Comp. Physiol., 35, 39-73.

Nelson, T.E. (1973) Porcine Stress Syndrome. In Malignant Hyperthermia, pp 191-197. Gordon, R.A., Britt, B.A. and Kalow, W. (eds). Proceedings of the International Symposium held in Toronto, 1971. Charles C. Thomas, Springfield, Illinois, U.S.A.

Nelson, T.E. (1978) Temperature effects on malignant hyperthermia skeletal muscle: diagnostic and etiologic implications. Abstracts of Scientific Papers, American Society of Anesthesiologists, p557.

Nelson, T.E. (1983) Abnormality in calcium release from skeletal sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia. J. Clin. Invest., 72, 862-870.

Nelson, T.E., Austin, K.L. and Denborough, M.A. (1977) Screening for malignant hyperpyrexia. Br. J. Anaesth., 49, 169-172.

Nelson, T.E. and Denborough, M.A. (1977) Studies on normal human skeletal muscle in relation to the pathopharmacology of malignant hyperpyrexia. Clin. Exp. Pharmac. Physiol., 4, 315-322.

Nelson, T.E., Flewellen, E.H. and Arnett, D.W. (1983) Prolonged electromechanical coupling time intervals in skeletal muscle of pigs susceptible to malignant hyperthermia. Muscle and Nerve, 6, 263-268.

Nelson, T.E., Jones, E.W., Venable, J.H. and Kerr, D.D. (1972) Malignant hyperthermia of Poland China Swine: studies of a myogenic etiology. Anesthesiology, 36, 52-56.

Nelson, T.E. and Schochet, S.S. (1982) Malignant hyperthermia: a disease of specific myofibre type? Can. Anaesth. Soc. J., 29, 163-167.

Newton, D.L. and Klee, C.B. (1984) CAPP-calmodulin: a potent competitive inhibitor of calmodulin actions. Febs Letters, 165, 269-272.

Niebroj-Dobosz, I. and Mayzner-Zawadska, E. (1982) Experimental porcine malignant hyperthermia: the activity of certain transporting enzymes and myofibrillar calcium-binding protein content in the muscle fibre.
Br. J. Anaesth., 54, 885-891.

Nie, N.H., Hull, C.H., Jenkins, J.G., Steinbrenner, K. and Bent, D.H. (1970) Statistical Package for the Social Sciences.
McGraw-Hill, New York.

Nissen, R.L. and Yonkers, A.J. (1982) Malignant Hyperthermia.
Laryngoscope, 92, 1183-1186.

Norman, J.A., Drummond, A.H. and Moser, P. (1979) Inhibition of calcium-dependent regulator-stimulated phosphodiesterase activity by neuroleptic drugs is unrelated to their clinical efficacy.
Mol. Pharmacol., 16, 1089-1094.

Ogawa, Y. (1970) Some properties of fragmented frog sarcoplasmic reticulum with particular reference to its response to caffeine.
J. Biochem. (Tokyo), 67, 667-683.

Ohnishi, S.T., Taylor, S. and Gronert, G.A. (1983) Calcium-induced Ca^{2+} release from sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia. The effects of halothane and dantrolene.
Febs Letters, 162, 103-107.

Ohtsuki, I. (1975) Distribution of troponin components in the thin filament studied by immunoelectronmicroscopy.
J. Biochem., 77, 633-639.

Okumura, F., Crocker, B.D. and Denborough, M.A. (1979) Identification of susceptibility to malignant hyperpyrexia in swine.
Br. J. Anaesth., 51, 171.

Okumura, F., Crocker, B.D. and Denborough, M.A. (1980) Site of the muscle cell abnormality in swine susceptible to malignant hyperpyrexia. Br. J. Anaesth., 52, 377-383.

Ono, K., Topel, D.G. and Althen, T.G. (1976) Cyclic AMP in longissimus muscle from control and stress susceptible pigs.
J. Food Sci., 41, 108-110.

Ono, K., Topel, D.G. and Althen, T.G. (1977) Adenylate cyclase and cyclic 3',5'-nucleotide phosphodiesterase activities in muscles from stress-susceptible and control pigs.
J. Food. Sci., 42, 111-112.

Oppenheim, G. (1973) Mutism and hyperthermia in a patient treated with neuroleptics.
Med. J. Aust., 2, 228-229.

Otswald, T.J. and MacLennan, D.H. (1974) Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum.
J. Biol. Chem., 249, 974-979.

Perrie, W.T., Smillie, L.B. and Perry, S.V. (1973) A phosphorylated light-chain component of myosin from skeletal muscle.
Biochem. J., 135, 151-164.

Perry, S.W., Grand, R.J.A., Nairn, A.C., Vanaman, T.C. and Wall, C.M. (1979) Metal ions and their influence on protein structure and function.
Biochem. Soc. Trans., 7, 619-622.

Peterson, G.L. (1977) A simplification of the protein assay method of Lowry et al which is more generally applicable.
Anal. Biochem., 83, 346-356.

Potter, J.D. and Gergely, J. (1974) Troponin, tropomyosin, and actin interactions in the Ca^{2+} regulation of muscle contraction.
Biochemistry, 13, 2697-2703.

Prozialeck, W.C., Cimino, M. and Weiss, B. (1981) Photoaffinity labeling of calmodulin by phenothiazine antipsychotics.
Mol. Pharmacol., 19, 264-269.

Rasmussen, H. and Waisman, D.M. (1983) Modulation of cell function in the calcium messenger system.
Rev. Physiol. Biochem. Pharmacol., 95, 111-148.

Reid, R.E., Gariepy, J. and Hodges, R.S. (1983) Interaction of neuroleptic drugs with a synthetic calcium-binding peptide analog of site III of rabbit skeletal troponin C. Phenothiazine selective binding.
Febs Letters, 154, 60-64.

Rosenberg, H., Fisher, C.A., Reed, S.B. and Addonizio, V.P. (1981) Platelet Aggregation in Patients susceptible to Malignant Hyperthermia.
Anesthesiology, 55, 621-624.

Roufogalis, B.D. (1979) Regulation of calcium translocation across the red blood cell membrane.
Can. J. Physiol. Pharmacol., 57, 1331-1349.

Roufogalis, B.D. (1980) Calmodulin: its role in synaptic transmission.
Trends in Neurological Science, 3, 238-241.

Roufogalis, B.D. (1981) Phenothiazine antagonism of calmodulin: a structurally-nonspecific interaction.
Biochem. Biophys. Res. Commun., 98, 607-613.

Roufogalis, B.D. (1982) Specificity of trifluoperazine and related phenothiazines for calcium binding proteins. In Calcium and Cell Function, Vol. 3, Cheung, W.Y. (ed), pp 129-159. Academic Press, New York.

Roufogalis, B.D., Minocherhomjee, A-E-V.M. and Al-Jobore, A. (1983) Pharmacological antagonism of calmodulin. Can. J. Biochem. Cell Biol., 61, 927-933.

Salama, G. and Scarpa, A. (1983) Mode of action of diethyl ether on ATP-dependent Ca^{2+} transport by sarcoplasmic reticulum vesicles. Biochem. Pharmacol., 32, 3465-3477.

Sarquist, F. and Larson, C.P. (1973) Drug-induced heat stroke. Anesthesiology, 39, 348-350.

Scarlett, J.D., Zimmerman, R. and Berkovic, S.F. (1983) Neuroleptic malignant syndrome. Aust. NZ J. Med., 13, 70-73.

Scharff, O. (1981) Calmodulin - and its role in cellular activation. Cell Calcium, 2, 1-27.

Schatzman, R.C., Wise, B.C. and Kuo, J.F. (1981) Phospholipid-sensitive calcium-dependent protein kinase: inhibition by antipsychotic drugs. Biochem. Biophys. Res. Commun., 98, 669-676.

Schmitt, J., Schmidt, K. and Ritter, H. (1974) Hereditary malignant hyperpyrexia associated with muscle adenylate kinase deficiency. Hum. Genet., 24, 253-257.

Schneider, M.F. and Chandler, W.K. (1973) Voltage dependent charge movement in skeletal muscle : a possible step in excitation-contraction coupling. Nature, 242, 244-246.

Schulman, H. and Greengard, P. (1978) Ca^{2+}dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium-dependent regulator". Proc. Nat. Acad. Sci. (U.S.A.), 75, 5432-5436.

Seeman, P. (1972) The membrane actions of anesthetics and tranquilizers. Pharmacol. Rev., 24, 583-655.

Seeman, P. (1977) Anti-schizophrenic drugs - membrane receptor sites of action.
Biochem. Pharmacol., 26, 1741-1748.

Sharma, R.K. and Wang, J.H. (1979) Preparation and assay of the Ca^{2+}-dependent modulator protein.
Adv. Cyclic Nucl. Res., 10, 187-198.

Sharma, R.K. and Wang, J.H. (1981) Inhibition of calmodulin-activated cyclic nucleotide phosphodiesterase by Triton X-100.
Biochem. Biophys. Res. Commun., 100, 710-715.

Short, C.E. and Paddleford, R.R. (1973) Malignant hyperthermia in the dog.
Anesthesiology, 39, 462-463.

Sjostrom, M. and Squire, J.M. (1977) The fine structure of the A-band in cryo-sections.
J. Mol. Biol., 109, 49.

Smego, R.A. and Durack, D.T. (1982) The neuroleptic malignant syndrome. Arch. Intern. Med., 142, 1183-1185.

Smoake, J.A., Song, S.-Y. and Cheung, W.Y. (1974) Cyclic 3',5'-nucleotide phosphodiesterase. Distribution and developmental changes of the enzyme and its protein activator in mammalian tissues and cells.
Biochim. Biophys. Acta, 341, 402-411.

Snyder, H.R., Davis, C.S., Bickerton, R.K. and Halliday, R.P. (1967) 1-[(5-arylfurfurylidine)] amino hydantoins. A new class of muscle relaxants.
J. Med. Chem., 10, 807-810.

Solomons, C.C., McDermott, N. and Mahowald, M. (1980) Screening for malignant hyperthermia with a platelet bioassay. New Eng. J. Med., 303, 642.

Sparrow, M.P., Mrwa, U., Hofmann, F. and Ruegg, J.C. (1981) Calmodulin is essential for smooth muscle contraction. Febs Letters, 125, 141-145.

Stauder, K.H. (1934) Die todliche katatonie. Arch. Psychiatr. Nervenkr., 102, 614-634.

Sullivan, J.S. and Denborough, M.A. (1981) Temperature dependence of muscle function in malignant hyperpyrexia-susceptible swine. Br. J. Anaesth., 53, 1217-1222.

Sullivan, J.S., Galloway, G.J. and Denborough, M.A. (1983) The effect of the calcium antagonist 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxy-benzoate on malignant hyperpyrexia-susceptible porcine skeletal muscle. Clin. Exp. Pharmacol. Physiol., 10, 587-593.

Takagi, A., (1981) Chlorpromazine and skeletal muscle: a study of skinned single fibres of the guinea pig. Exp. Neurol., 73, 477-486.

Teo, T.S., Wang, T.H. and Wang, J.H. (1973) Purification and properties of the protein activator of bovine heart cyclic adenosine 3',5'-mono-phosphate phosphodiesterase. J. Biol. Chem., 248, 588-595.

Tollefson, G. (1982) A case of neuroleptic malignant syndrome: *in vitro* muscle comparison with malignant hyperthermia. J. Clin. Psychopharmacol., 2, 266-270.

Topel, D.G., Bricknell, E.J., Preston, K.S., Christian, L.L. and Matsushima, C.Y. (1968) Porcine stress syndrome. Mod. Vet. Pract., 49, 40-41 and 59-60.

Traynor, C.A., Van Dyke, R.A. and Gronert, G.A. (1983) Phosphorylase ratio and susceptibility to malignant hyperthermia. Anesth. Analg., 62, 324-326.

Van den Hende, C., Lister, D., Muylle, E., Ooms, L. and Oyaert, W. (1976) Malignant hyperthermia in Belgian landrace pigs rested or exercised before exposure to halothane. Br. J. Anaesth., 48, 821-829.

Verbeeck, R.K., Cardinal, J.-A., Hill, A.G. and Midha, K.K. (1983) Binding of phenothiazine neuroleptics to plasma proteins. Biochem. Pharmacol., 32, 2565-2570.

Vincenzi, F.F. (1979) Calmodulin in the regulation of intracellular calcium. Proc. Western Pharmacol. Soc., 22, 289-294.

Vincenzi, F.F., Adunyah, E.S., Niggli, V. and Carafoli, E. (1982) Purified red blood cell Ca^{2+}-pump ATPase: evidence for direct inhibition by presumed anti-calmodulin drugs in the absence of calmodulin. Cell Calcium, 3, 545-559.

Wang, J.H., Tam, S.W., Lewis, W.G. and Sharma, R.K. (1981) The role of calmodulin in the regulation of protein phosphorylations in skeletal muscle. Cold Spring Harbour Conferences on Cell Proliferation, 8(A), 357-371.

Wang, J.H. and Waisman, D.M. (1979) Calmodulin and its role in the second-messenger system. In Current Topics in Cellular Regulation, vol. 15, pp 47-107. Academic Press, New York.

Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3',5'-cyclic nucleotide phosphodiesterase and actomyosin ATPase.
J. Biol. Chem., 251, 4501-4513.

Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980) The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain.
J. Biol. Chem., 255, 962-971.

Weber, A. (1968) The mechanism of the action of caffeine on sarcoplasmic reticulum.
J. Gen. Physiol., 52, 760-772.

Weber, A. and Herz, R. (1968) The relationship between caffeine and contracture of intact muscle and the effect of caffeine on reticulum.
J. Gen. Physiol., 52, 750-759.

Weiss, B., Fertel, R., Figlin, R. and Uzunov, P. (1974) Selective alteration of the activity of the multiple forms of adenosine 3',5'-monophosphate phosphodiesterase of rat cerebrum.
Mol. Pharmacol., 10, 615-625.

Weiss, B., Prozialeck, W., Cimino, M., Barnette, M.S. and Wallace, T.L. (1980) Pharmacological regulation of calmodulin.
Ann. N.Y. Acad. Sci., 356, 319-345.

Weiss, B., Prozialeck, W.C. and Wallace, T.L. (1982) Interaction of drugs with calmodulin: biochemical, pharmacological and clinical implications.
Biochem. Pharmacol., 31, 2217-2226.

Weinberger, D.R. and Kelly, M.J. (1977) Catatonia and malignant syndrome: a possible complication of neuroleptic administration. J. Nerv. Ment. Dis., 165, 263-268.

Weinberg, S. and Twersky, R.S. (1983) Neuroleptic malignant syndrome. Anesth. Analg., 62, 848-850.

White, G.C. and Raynor, S.T. (1980) The effects of trifluoperazine, an inhibitor of calmodulin, on platelet function. Thrombosis Research, 18, 279-284.

White, M.D., Collins, J.G. and Denborough, M.A. (1983) The effect of dantrolene on skeletal-muscle sarcoplasmic-reticulum function in malignant hyperpyrexia in pigs. Biochem. J., 212, 399-405.

White, M.D., Thomas, C.R. and Denborough, M.A. (1983). A novel method for the isolation of calsequestrin from porcine skeletal muscle sarcoplasmic reticulum. Biochim. Biophys. Acta, 744, 1-6.

Williams, C.H. (1976) Some observations on the etiology of the fulminant hyperthermia-stress syndrome. Perspect. Biol. Med., 20. 120-130.

Willner, J. (1984) Phosphorylase activity and malignant hyperthermia. Anesth. Analg., 63, 88-89.

Willner, J.H., Cerri, C.G. and Wood, D.S. (1981) High skeletal muscle adenylate cyclase in malignant hyperthermia. J. Clin. Invest., 68, 1119-1124.

Willner, J.H., Wood, D.S., Cerri, C. and Britt, B. (1980) Increased myophosphorylase A in malignant hyperthermia. New Eng. J. Med., 303, 138-140.

Wolff, D.J., Poirier, P.G., Brostrom, C.D. and Brostrom, M.A. (1977) Divalent cation binding properties of bovine brain Ca^{2+}-dependent regulator protein.
J. Biol. Chem., 252, 4108-4117.

Wong, P.Y.-K. and Cheung, W.Y. (1979) Calmodulin stimulates human platelet phospholipase A_2.
Biochem. Biophys. Res. Commun., 90, 473-480.

Yagi, K., Yazawa, M., Kakiuchi, S., Oshima, M. and Uenishi, K. (1978) Identification of an activator protein for myosin light chain kinase as the Ca^{2+}-dependent modulator protein.
J. Biol. Chem., 253, 1338-1340.

Yamauchi, T. and Fujisawa, H. (1979a) Activation of tryptophan 5-mono-oxygenase by calcium-dependent regulator protein.
Biochem. Biophys. Res. Commun., 90, 28-35.

Yamauchi, T. and Fujisawa, H. (1979b) Most of the Ca^{2+}-dependent endogenous phosphorylation of rat brain cytosol proteins requires Ca^{2+}-dependent regulator protein.
Biochem. Biophys. Res. Commun., 90, 1172-1178.

Yasawa, M., Sakuma, M. and Yagi, K. (1980) Calmodulins from muscles of marine invertebrates, scallop and sea anemone. Comparisons with calmodulins from rabbit skeletal muscle and pig brain.
J. Biochem. (Tokyo), 87, 1313-1320.